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13. ABSTRACT ***(Maximum 200 Words)***

Neuronal and astrocyte cell cultures prepared from the cerebellum, cortex, or midbrain of wild-type or DNA repair-deficient (i.e., $MGMT^{-/-}$, $AAG^{-/-}$, $XPA^{-/-}$) mice were examined for the acute toxicity to nitrogen mustard (HN2) or the related alkylating agent methylazoxymethanol (MAM). Cerebellar neurons were more sensitive to MAM and HN2 than astrocytes or neurons from other brain regions (i.e., cortex and midbrain). This sensitivity to MAM and HN2 was accompanied by markers of apoptosis and DNA damage (i.e., single-strand breaks). In comparably treated cultures from DNA repair-deficient mice, cell survival was preserved in $AAG^{-/-}$ neurons, but reduced in $MGMT^{-/-}$ and $XPA^{-/-}$ neurons treated with HN2 or MAM. Unexpectedly, neuronal survival was significantly reduced in $MGMT^{-/-}$ cerebellar neurons treated with HN2. Similar results were also obtained for comparably treated fibroblast cell lines from all three DNA repair-deficient mice. However, the long-term survival of fibroblasts were only reduced in $MGMT^{-/-}$ and $XPA^{-/-}$ fibroblasts treated with HN2 or MAM. Loss of cerebellar neurons, degeneration and DNA damage were also observed in wild type mice administered MAM. *In vitro* and *in vivo* studies are currently underway with DNA repair-deficient mice to further examine the relationship between DNA damage and the delayed neurotoxicity of MAM and HN2.

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G. Kisby

PI - Signature Date

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INTRODUCTION

Experiments are proposed to examine the molecular mechanism by which mustard chemical warfare agents induce neuronal cell death. DNA damage is the proposed underlying mechanism of mustard-induced neuronal cell death. We propose a novel research strategy to test this hypothesis by using mice with perturbed DNA repair to explore the relationship between mustard-induced DNA damage and neuronal cell death. Initial *in vitro* studies (Years 1, 2 & 3) are proposed to examine the cytotoxic and DNA damaging properties of the sulfur mustard analogue mechlorethamine (nitrogen mustard or HN2) and the neurotoxic DNA-damaging agent methylazoxymethanol (MAM) using neuronal and astrocyte cell cultures from different brain regions of mice with perturbed DNA repair. Findings from these studies will be used to examine the *in vivo* neurotoxic effects of HN2 and MAM (Years 3 & 4) in mice with perturbed DNA repair.

BODY OF THE REPORT

STATEMENT OF WORK FOR YEAR 3 of FUNDING

The overall goal of studies proposed in Year 3 are to: (i) examine the cytotoxicity of HN2 and MAM in neuronal and astrocyte cell cultures of DNA repair-mutant mice, (ii) examine HN2 and MAM treated neuronal and astrocyte cell cultures of DNA repair-mutant mice for DNA damage, and (iii) use results from these *in vitro* studies to begin dose-range finding studies of HN2 and MAM in wild type and DNA repair deficient mice. Specific objectives proposed in Year 3 of the Statement of Work are as follows:

1. Examine cytotoxicity of HN2 and MAM in MGMT-deficient neuronal and astrocyte cell cultures.
2. Examine cytotoxicity of HN2 and MAM in AAG-deficient neuronal and astrocyte cell cultures.
3. Examine cytotoxicity of HN2 and MAM in XPA-deficient neuronal and astroctye cell cultures.
4. Measure DNA damage of HN2 and MAM in MGMT-deficient neuronal and astrocyte cell cultures.
5. Measure DNA damage of HN2 and MAM in AAG-deficient neuronal and astrocyte cell cultures
6. Measure DNA damage of HN2 and MAM in XPA-deficient neuronal and astrocyte cell cultures
7. Commence dose-range finding studies of HN2 and MAM in MGMT- and AAG-deficient mice.

For the ongoing studies, we are currently breeding four strains of mice that either overexpress (1 strain) or are deficient (3-strains) in three key proteins of different DNA repair pathways. These are XPA, MGMT (1-overexpression, 1-knock-out), and AAG. Despite significant problems encountered in the first two years of the grant, we were able to get back on 'track' and complete most of the objectives proposed in Year 3 of the grant. We attribute this success to an increased breeding strategy and the efficient genotyping of mice by two highly trained technicians, which provided a sufficient number of animals to meet a majority of the proposed objectives. Despite these aggressive efforts (2 neuronal and/or astrocyte cell cultures/every other week), we were not able to generate a sufficient number of animals to complete both the acute (24h) and delayed (3d – 14d) cytotoxicity and DNA damage studies. Moreover, we needed to establish skin fibroblast cell cultures for each strain to clarify the acute neurotoxic properties of HN2 and MAM in the three different DNA repair genotypes (i.e., $MGMT^{-/-}$, $AAG^{-/-}$, $XPA^{-/-}$). Consequently, studies in Year 3 focused on examining the <u>acute</u> cytotoxic and DNA damage properties of HN2 and MAM in neurons, astrocytes and fibroblasts. We were successful in examining all of the objectives of the Statement of Work (except for # 4) in Year 3 by focusing on: (i) the comparative toxicity of HN2 and MAM in neuronal and astrocyte cell cultures from different brain regions (i.e., cerebellum, cortex and midbrain) of wild-type mice, (ii) the comparative toxicity of HN2 and MAM in nervous and non-nervous tissue (i.e., fibroblasts) of wild type and DNA repair deficient

mice, (iii) the extent and type of DNA damage induced by HN2 and MAM in neuronal and fibroblast cultures from wild type and DNA repair deficient mice, and (iv) the extent of neuronal loss, neurodegeneration, and DNA damage induced by MAM in the brains of wild type mice. A detailed description of the research accomplishments for each objective of Year 3 follows.

1. Examine cytotoxicity of HN2 and MAM in wild type and MGMT-deficient neuronal and astrocyte cell cultures.

Wild Type Studies

Cytotoxicity

The colony of wild-type (i.e., C57BL6) mice that we established during the 1st year of study was used primarily during the 2nd and 3nd years to: (i) expand our colonies of *Aag*-, *Mgmt*-, and *Xpa*-mutant mice for developing cell cultures from brain tissue of both postnatal (cerebellum) and embryonic (cortex and midbrain) animals, (ii) examine cell cultures of cortex, midbrain, and cerebellar neurons and astrocytes for HN2 and MAM-induced cytotoxicity, and (iii) examine the *in vivo* neurotoxic and DNA damaging properties of HN2 and MAM.

Studies conducted in Year 2 of the grant suggested that mice which overexpress MGMT are partially protected from the acute toxicity of MAM (24h), but not HN2 (*see draft manuscript in* Appendix). Comparative studies also demonstrated that wild type and $MGMT^+$ neurons were more sensitive to low concentrations of HN2 (1.0 μM) and MAM (100 μM) than astrocytes. This selective vulnerability of CNS neurons (*vs.* astrocytes) to MAM and HN2 was confirmed by further examining the viability of wild type cerebellar neurons and astrocytes to a wide range of both toxins (*see* **Figure 1,** Appendix). The viability of wild type neurons was significantly lower at all concentrations of MAM (100 μM-1000 μM) and HN2 (1-20 μM) than comparably treated astrocytes. Since these studies demonstrated that wild type astrocytes were essentially insensitive to both MAM and HN2, the remaining studies in Year 3 focused primarily on the neurotoxic properties of these alkylating agents.

The brain is composed of various regions and, therefore, neurons within these regions may exhibit differences in their sensitivity to MAM or HN2. Similar studies were conducted in neuronal cultures from different brain regions to determine if the acute toxic properties of MAM and HN2 differs among CNS neurons (*see* **Figure 2,** Appendix). Mature neuronal cultures prepared from the postnatal cerebellum or embryonic cortex and midbrain of wild type mice were treated for 24h with various concentrations of MAM (10 μM-2000 μM) or HN2 (0.1 μM-20 μM) and examined for cell viability by measuring redox function (Alamar Blue) or the live/dead assay (calcein AM/propidium iodide). Overall, cerebellar neurons were significantly more sensitive to MAM than similarly treated midbrain or cortical neurons. However, cortical and cerebellar neurons were more sensitive to high concentrations of HN2 (>10 μM) than midbrain neurons. These studies demonstrate that neurons from various brain regions exhibit differences in their senstivity to MAM and HN2, an effect possibly related to differences in DNA repair. Studies are currently underway to examine the basal expression of various DNA repair proteins (i.e., MGMT, AAG, XPA) in neurons and astrocytes from these brain regions.

Taken together, these findings suggest that neurons are a key target (*vs.* astrocytes) of MAM and HN2-induced toxicity and this selective vulnerability extends to neurons within certain brain regions. Since the acute toxic effects of MAM and HN2 were more pronounced in cerebellar neurons, these neuronal cultures were used throughout the remainder of the acute toxicity studies in Year 3. However, comparable long-term studies are currently underway with cerebellar, cortical and midbrain neuronal

cultures from each strain (i.e., $MGMT^{-/-}$, $AAG^{-/-}$, and $XPA^{-/-}$) to determine if these cells also differ in their sensitivity to the delayed effects of MAM and HN2.

The underlying mechanism of neuronal cell death induced by MAM or HN2 was further investigated by examining toxin treated wild type cerebellar neurons for markers of apoptosis, a suicide cellular process for the removal of damaged or injured cells. Apoptosis is an active, genetically regulated cellular mechanism that involves the loss of mitochondrial membrane permeability and nuclear alterations (chromatin condensation followed by fragmentation) that culminate in the destruction of damaged cells. The metabolic pathways involved in apoptosis have been elucidated and a variety of well-established techniques are used to examine cells for early, active and late apoptotic events. We used a combination of these techniques to examined MAM and HN2 treated wild type neurons for well-known markers of different periods of apoptosis, that is, loss of the mitochondrial membrane potential psi (an early event), the activation of cellular caspases (a cysteine proteases; a later event) and DNA fragmentation (TUNEL labelling; a late-stage event). Wild type cerebellar neurons were treated with various concentrations of MAM or HN2 for 24h and examined for mitochondrial membrane potential (DePsipher™, Trevigen, Inc), the activation of multiple cellular caspases (CaspaTag™, Intergen)(*see* **Figure 3,** Appendix) and DNA fragmentation (NeuroTacs™, Trevigen, Inc) (*see* **Figure 4,** Appendix). The mitochondrial membrane potential (delta psi) of wild type cerebellar neurons was preserved (*punctate orange-red aggregates*) at most MAM concentrations, but was severely perturbed (*diffuse cytoplasmic green fluoresence*) at high concentrations of MAM (>100 μM). In contrast, low concentrations of HN2 (>1.0 μM) induced significant loss of the mitochondrial membrane potential suggesting that mechanism of neuronal death induced by HN2 differs from that of MAM. However, neither MAM (*see* **Figure 3**, *middle panels*) or HN2 (*data not shown*) induced a significant activation of neuronal caspases suggesting that the mechanism of acute neuronal cell death by these agents is not solely apoptotic. Consistent with this hypothesis, DNA fragmentation (*see* **Figure 4,** Appendix) was significantly elevated ($p<0.001$ *or* $p<0.01$, respectively) only in cerebellar neurons treated with high concentrations of either MAM (1000 μM) or HN2 (5.0 μM and 10 μM).

<u>$MGMT^{-/-}$ Studies</u>

Our previous studies indicate that MGMT-overexpressing neurons were partially protected from the acute toxic effects of MAM, but not HN2. Therefore, if O^6-methylguanine DNA adducts are important in the acute neurotoxicity of MAM then MGMT-deficient ($MGMT^{-/-}$) neurons should be especially more sensitive to MAM than HN2. To test this hypothesis, MGMT deficient ($MGMT^{-/-}$) cerebellar neurons were treated with similar concentrations of HN2 and MAM (*see* **Figure 5,** Appendix) and examined for cell viability by measuring mitochondrial function (Alamar Blue) or the live/dead assay (calcein AM/propidium iodide). Overall, $MGMT^{-/-}$ cerebellar neurons appeared more sensitive to the acute toxic effects of both MAM and HN2 than wild type cells, but only at very high concentrations of MAM (i.e., >1000 μM). Unexpectedly, $MGMT^{-/-}$ cerebellar neurons were more sensitive to the acute toxic effects of HN2 than wild type cells, especially at relatively low concentrations (5 μM or greater) of the mustard agent. Since HN2 is known not to form O^6-methylguanine DNA adducts, these findings suggest that perturbing cellular MGMT may disturb the balance of repair for HN2-induced DNA damage. These findings suggest that O^6-methylguanine DNA adducts do not appear to play an important role in the acute neurotoxicity of MAM or HN2. Therefore, the protection we found for MGMT overexpressing neurons may be mediated by a different mechanism, possibly by the influence of MGMT on other DNA repair pathways (e.g., base-excision or nucleotide excision DNA repair pathways). Alternatively, these adducts may play a more important role in the delayed or chronic neurotoxic effects of MAM. In support, we show later that $MGMT^{-/-}$ mouse ear fibroblasts treated acutely with similar concentrations of

MAM are significantly more sensitive to MAM than comparably treated wild-type ear fibroblasts (*see* **Figure 8,** Appendix) and that MAM-induced DNA damage varies considerably among neurons with different DNA repair capacity (i.e., $AAG^{-/-}$- vs. $XPA^{-/-}$; *see* **Figure 10,** Appendix). Studies are now underway to determine if similar treatments of neurons with MAM (or HN2) increase specific types of DNA damage other than strand breaks (e.g., O^6-mdG, N^7-alkyldG, AP sites) that may ulitimately lead to a delayed toxic effect of these agents in $MGMT^{-/-}$ cells. Moreover, $MGMT^{-/-}$ mice will be administered MAM or HN2 to determine if the delayed neurotoxic effects of these agents on cerebellar granule cells also occurs *in vivo*.

2. Examine cytotoxicity of HN2 and MAM in AAG-deficient neuronal and astrocyte cell cultures. Findings from the above $MGMT^{-/-}$ and previous $MGMT^{+}$ studies suggest that O^6-methylguanine DNA adducts do not appear to play a major role in the acute neurotoxicity of MAM or HN2. However, MAM and HN2 induce multiple DNA adducts including N^7-methylguanine (N^7-mGua), 8-methylguanine or N^7-alkylguanine DNA adducts, respectively. Since N^7-mGua and N^7-alkylguanine DNA adducts are the predominant DNA adducts produced by MAM or HN2 and alkylguanine DNA glycosylase (AAG) is the primary protein that repairs these types of DNA adducts, primary cultures of $AAG^{-/-}$ cerebellar neurons were treated with MAM or HN2 to determine if these DNA adducts have a role in the acute neurotoxicity of HN2 or MAM. To test this hypothesis, cerebellar neurons from $AAG^{-/-}$ mice were plated at the same density (140-150K cells/well) and treated for 24h with the same concentrations of MAM (10 μM-2000 μM) or HN2 (0.1 μM-20 μM) as neurons from $MGMT^{+}$ or $MGMT^{-/-}$ mice. After toxin treatment, the viability of the cells was examined by measuring mitochondrial function (Alamar Blue™) or cell survival (calcein AM/propidium iodide) (*see* **Figure 6,** Appendix). Unlike $MGMT^{-/-}$ neurons, $AAG^{-/-}$ cerebellar neurons were less sensitive than comparably treated wild-type cerebellar neurons to the acute neurotoxic effects of MAM or HN2. Moreover, $AAG^{-/-}$ cerebellar neurons appeared to be resistant to high concentrations of both MAM (1000 μM and 2000 μM) and HN2 (5-20 μM) as indicated by the preservation of mitochondrial function (*top figures*). These studies suggest that N^7-methylguanine or N^7-alkylguanine DNA adducts are probably not responsible for the acute neurotoxicity of either MAM or HN2. However, these DNA adducts may play a more important role in the delayed neurotoxicity induced by either MAM or HN2 because, later, we show that DNA damage (i.e., AP sites) was elevated in $AAG^{-/-}$ neurons treated with concentrations of MAM (10 μM and 100 μM) or HN2 (10 μM) that were not acutely toxic to $AAG^{-/-}$ neurons (*see* **Figure 10,** Appendix). Moreover, we observed very little DNA damage (i.e., strand breaks, AP sites) (*see* **Figures 9 & 10,** Appendix) or changes in the long-term survival of comparably treated $AAG^{-/-}$ fibroblasts suggesting that these type of DNA adducts persist in post-mitotic cells and result in delayed neurotoxicity. Comparable long-term studies with $MGMT^{-/-}$ and $AAG^{-/-}$ cerebellar neurons are currently underway to examine the relationship between certain types of DNA damage and the delayed neurotoxic effects of MAM and HN2.

3. Examine cytotoxicity of HN2 and MAM in XPA-deficient neuronal and astrocyte cell cultures. The *Xpa* gene codes for a NER protein that is responsible for recognizing bulky adducts and x-links [**2**]. The cytotoxic properties of HN2 and MAM was examined in cerebellar neuronal cultures prepared from wild-type (C57BL/6) mice and crosses of $XPA^{-/-}$ mice. Wild-type and $XPA^{-/-}$ neuronal cultures were treated for 24h with control culture media or culture media containing various concentrations of HN2 (0.1 μM -10 μM) or methylazoxymethanol (MAM; 50 μM – 1000 μM) (*see* **Figure 7,** *Appendix*). Viability was determined in HN2 and MAM treated neuronal cell cultures by adding Alamar Blue™, a non-toxic metabolic indicator that is widely used to measure mitochondrial function in different cell systems (including neurons) [**9,10**] and the number of surviving cells quantified by staining the cells

with the vital fluorescent probe calcein-AM. Like MGMT$^{-/-}$ cerebellar neurons, XPA$^{-/-}$ neurons were very sensitive to high concentrations of MAM (>1000 μM). In contrast to MGMT$^{-/-}$ neurons, XPA neurons were equally sensitive as wild type neurons to HN2. However, mitochondrial activity was significantly higher in HN2 or MAM treated XPA$^{-/-}$, AAG$^{-/-}$ and MGMT$^{-/-}$ neurons cells than comparably treated wild type cells suggesting that these toxins do not influence mitochondrial function. Altogether, these findings suggest that XPA$^{-/-}$ and MGMT$^{-/-}$ neurons are inefficient at removing DNA damage induced by MAM or HN2 (respectively) when compared with similarly treated AAG$^{-/-}$ cells. To confirm this hypothesis, XPA$^{-/-}$ and AAG$^{-/-}$ neuronal cultures were treated for 24h with MAM (10 μM or 100 μM) or HN2 (10 μM) and examined for DNA damage (*see* **Figure 10**, Appendix). We used a method recently developed by Atamna *et al.* [**1**] to detect AP sites in living cells by incubating live neuronal cultures with 1.0 mM ARP (a biotinylated aldehyde reactive probe), an agent that penetrates the plasma membrane of cells and reacts with AP sites in DNA to form stable ARP-DNA adducts. AP sites were detected by incubating ARP treated neuronal cultures with CY3-conjugated streptavidin and fluorescence microscopy. The level of apurinic/apyrimidinic (AP) sites (*red fluoresecence*) was significantly higher in XPA$^{-/-}$ neurons treated with 10 μM MAM and 100 μM MAM than comparably treated AAG$^{-/-}$ neurons. More importantly, the detection of significant levels of AP sites at non-lethal concentrations of MAM in both XPA and AAG neurons and fibroblasts suggests that this type of DNA adducts persist in post-mitotic neurons. These DNA adducts may be responsible for the delayed neurotoxic effects of MAM. Comparable studies are currently underway in wild type and MGMT$^{-/-}$ neurons to determine if the cytotoxic properties of MAM or HN2 are also related to the extent of AP sites. Results from these studies will be compared with the extent of cytotoxicity and the level of other DNA adducts (e.g., O^6-methyldeoxyguanosine, N^7-mdG, N^7-alkylguanine) to whether specific DNA adducts are resposible for MAM or HN2-induced neurotoxicity.

4. Measure DNA damage in HN2 and MAM treated MGMT-deficient neuronal and astrocyte cell cultures.

The primary hypothesis under study in this grant is that DNA damage plays an important role in neuronal cell death. The above cytotoxicity studies demonstrate that neurons with reduced DNA repair capacities (i.e., MGMT$^{-/-}$ and XPA$^{-/-}$) are acutely sensitive to MAM or HN2 when compared with similarly treated wild type neurons. The basis for this increased sensitivity is proposed to be an increase in unrepaired damage to DNA induced by MAM or HN2. Since MAM and HN2 induced multiple types of DNA adducts, we were interested in determining if these DNA adducts persist in injured post-mitotic neurons. To test this hypothesis, cerebellar neurons from MGMT$^{-/-}$ mice were treated with MAM or HN2 and the cells examined for different types of DNA damage including alkylated nucleobases (e.g., O^6-mdG, N^7-mdG), baseless sites (e.g., AP sites), strand breaks (i.e., nicks), or DNA fragmentation (e.g., apoptotic bodies). DNA repair deficient neurons exposed to MAM or HN2 would be expected to exhibit a wide range of DNA damage because of reduced repair of these DNA adducts. Since MGMT$^{-/-}$ neurons are particularly sensitive to HN2 (*see* **Figure 5**), the present studies compared the extent of DNA fragmentation (a late stage of apoptosis) induced by HN2 in wild type and MGMT$^{-/-}$ cerebellar neurons incubated with the bisbenzimide dye Hoescht 33342 (HO), a dye that fluoresces brilliant blue (*vs.* pale or azur blue) when it binds to damaged DNA (i.e., poly d (A-T) tracts) [**5**]. Extensive HO staining and the presence of apoptotic bodies was observed only at high concentrations of HN2 in MGMT$^{-/-}$ neurons, which is consistent with increased sensitivity of MGMT$^{-/-}$ neurons to high concentrations of HN2 (>5 μM). In contrast, HO staining was evident in wild type cerebellar neurons at lower concentrations of HN2 (>1.0 μM) and at high concentrations of MAM (>500 μM). HO staining of HN2 and MAM treated AAG$^{-/-}$ neurons (*data not shown*) was observed to be similar to that of

comparably treated wild type cells. These studies suggest that MGMT may be involved in HN2-induced apoptosis. Additional studies are underway to confirm these findings by examining HN2 and MAM treated $MGMT^{+}$ and $MGMT^{-/-}$ neurons for HO staining or other types of DNA damage (i.e., AP sites, damaged nucleobases).

5. Measure DNA damage in HN2 and MAM treated AAG-deficient neuronal and astrocyte cell cultures.

MAM and HN2 induce alkylpurines in DNA (i.e., N^7-methyl- and N^7-alkyl) and these DNA adducts are repaired by the base-excision DNA repair pathway. These DNA adducts are first removed by 3-methyladenine DNA glycosylase (AAG) to produce an abasic site (or AP site), which is subsequently repaired by AP endonuclease. Therefore, $AAG^{-/-}$ deficient neurons should be sensitive to both MAM and HN2 if these DNA adducts play an important role in neurotoxicity. However, acute cytotoxicity studies indicate that $AAG^{-/-}$ neurons are particularly insensitive to MAM and HN2 (*see* **Figure 6**, Appendix). Consequently, DNA damage would be expected to be comparable in similarly treated wild type and $AAG^{-/-}$ cerebellar neurons. To test this hypothesis, wild type and $AAG^{-/-}$ cerebellar neurons were treated for 24h with HN2 (0.1 μM, 1.0 μM, 10 μM) or MAM (10 μM, 100 μM, 1000 μM) and examined for the extent of AP sites by incubating the cells with ARP (a biotinylated aldehyde reactive probe; *see #3 for details*). Elevated levels of AP sites were detected in $AAG^{-/-}$ neurons treated with 10 μM HN2 and 100 μM MAM when compared with control treated $AAG^{-/-}$ neurons or lower concentrations of the toxins (*see* **Figure 10**, Appendix). More importantly, AP levels were significantly higher in $AAG^{-/-}$ neurons than comparably treated $AAG^{-/-}$ fibroblasts. Similar differences were also observed for the extent of DNA fragmentation (i.e., TUNEL labelling) in neurons and fibroblasts of wild type (*see* **Figure 4**, Appendix) and $AAG^{-/-}$ cerebellar neurons (*see* **Figure 9**, Appendix) treated with HN2 and MAM.

Taken together, these studies demonstrate that AP sites are more persistent in $AAG^{-/-}$ neurons, but the damage is not associated with acute neurotoxicity. The reduced survival of DNA repair deficient fibroblasts 2-3 weeks after a brief (24h) exposure to MAM suggests that DNA repair deficient neurons may be particularly sensitive to the delayed toxic effects of MAM or HN2. Studies are now underway to examine $AAG^{-/-}$ neurons and other DNA repair deficient neurons for the delayed neurotoxic effects (up to 14 days) induced by brief (24h) or continuous exposure to HN2 and MAM. These studies will help clarify the role of specific types of DNA damage in MAM and HN2-induced neurodegeneration.

6. Measure DNA damage in HN2 and MAM treated XPA-deficient neuronal and astrocyte cell cultures.

The *Xpa* gene codes for a nucleotide excision DNA repair (NER) protein that is responsible for recognizing bulky adducts and x-links [**3,7**]. Mustard-induced DNA damage is proposed to initiate tissue injury and cross-links are believed to be the principal lesion responsible for mustard-induced toxicity. Therefore, $XPA^{-/-}$ deficient neurons should be especially sensitive to HN2, but insensitive to MAM, which does not induced cross links or bulky DNA adducts. Like $AAG^{-/-}$ and $MGMT^{-/-}$ neurons, MAM and HN2 treated $XPA^{-/-}$ neurons were examined for DNA damage by measuring the extent of HO DNA fragmentation (HO staining), strand breaks (TUNEL labelling), and AP sites (ARP assay). $XPA^{-/-}$ neurons were treated with MAM (100 μM or 500 μM) and examined for HO staining (*see* **Figure 11**, Appendix). Strong staining of $XPA^{-/-}$ neurons was only observed at the highest concentration of MAM (i.e., 500 μM). However, $XPA^{-/-}$ cerebellar neurons were also strongly stained for AP sites at lower concentrations of MAM (10 μM and 100 μM) when compared with control treated $XPA^{-/-}$ neurons or similarly treated $XPA^{-/-}$ fibroblasts (*see* **Figure 10**, Appendix). Interestingly, these concentrations of

MAM were shown to be non-lethal to $XPA^{-/-}$ neurons (*see* **Figure 7**, Appendix). Staining for AP sites was also comparable in $XPA^{-/-}$ cerebellar neurons treated with 100 μM MAM or 10 μM HN2.

These studies demonstrate that AP sites are more persistent in HN2 and MAM treated $XPA^{-/-}$ neurons than comparably treated $XPA^{-/-}$ fibroblasts or $AAG^{-/-}$ neurons. The notion that the persistence of these DNA adducts may be associated with delayed neurotoxicity is supported by reduced long-term survival of fibroblasts from $MGMT^{-/-}$ or $XPA^{-/-}$ (but not $AAG^{-/-}$) mice after a brief exposure to MAM or HN2 (*see* **Figure 9**, Appendix). The difference in the level of AP sites among DNA repair deficient ($XPA^{-/-}$ *vs.* $AAG^{-/-}$) neurons suggests that certain types of DNA adducts (or repair pathways) may be more important in HN2 or MAM-induced neuronal cell death. Studies are now underway to examine $XPA^{-/-}$ neurons and other DNA repair deficient neurons for the delayed neurotoxic effects (up to 14 days) induced by brief (24h) or continuous exposure to HN2 and MAM. These studies will help clarify the role of specific types of DNA damage in MAM and HN2-induced neurodegeneration.

7. Commence dose-range finding studies of HN2 and MAM in *MGMT*-deficient and *AAG*-deficient mice.

Because of the difficulties experienced with the generation of sufficient DNA repair-deficient mice for the preparation of neuronal and astrocyte cell cultures for the cytotoxicity and DNA damage studies, dose-range finding studies proposed in Year 2 and 3 have been postponed for Year 4. Therefore, evidence from studies with the $MGMT^{+}$ and $MGMT^{-/-}$ mice conducted in Years 1-3 suggest that we should begin dose-range findings studies with these DNA repair mutant mice. $MGMT^{+}$ mice were obtained this spring (in collaboration with Dr. S. Gerson, Case Western) and a colony was established this summer after 3 breeder pairs were released from quarantine. Plans are already underway to commence dose-range findings studies with MAM and HN2 in $MGMT^{-/-}$ mice. Meanwhile, dose-range findings studies were conducted in wild type mice by treating 1-2 day old pups with a high dose of MAM (30 mg/kg, s.c.) to establish methods for assessing neuronal cell loss, neurodegeneration and DNA damage in MAM and HN2 treated DNA repair mutant mice. The dose and treatment age were chosen based upon several important factors: (i) the cerebellum (notably the granule cell layer) is severely compromised in mouse pups treated at 1-5 days with this concentration of MAM [**4**], (ii) we demonstrated that cultures of cerebellar neurons of wild type and $MGMT^{-/-}$ were especially sensitive to HN2,(*see above studies*) and (iii) $MGMT^{+}$ partially protects cultures of cerebellar granule cells from MAM-induced neurotoxicity. Two litters of C57Bl6 mice (n=8/litter) were administered either a single subcutaneous dose of MAM (30 mg/kg, n=4) in saline or a similar volume of saline (control, n=4). The animals were examined daily for changes in body weight and size (length from crown to rump), features that typically are reduced in MAM treated animals. As previously reported, the body weight of MAM treated animals was 20-25% lower than saline treated littermates and remained lower up until termination at day 21. Animals were perfused with 4% buffered paraformaldehyde and the brain and spinal cord cryoprotected in sucrose. Coronal brain tissue sections were made through the whole cerebellum, the serial sections stored at –90°C in cryoprotectant, and every tenth section examined for cerebellar morphology (cresyl violet or anti-calbindin-D staining), neurodegeneration (Fluoro-Jade B or silver staining) or DNA damage (TUNEL labelling). Calbindin-D is an intracellular calcium-binding protein that is especially abundant in Purkinje cells of the cerebellum and is, thus, was used as a marker for Purkinje cell degeneration. Fluoro-Jade B is a polyanionic fluorescein derivative useful for the histological staining of neurons undergoing neurodegeneration [**8**] while components of neurons undergoing degeneration (e.g., lysosomes, axons, terminals) become agryrophilic (affinity for silver ions) and upon reduction form dark grains that are visible by light microscopy. Gross observation of the

cerebellum from MAM treated mice revealed extensive atrophy of the cerebellar lobes (*stars*) when compared with the cerebellum of saline treated mice (*see* **Figure 12A**, Appendix). This was more evident in cresyl violet stained (*see* **Figure 12B**, Appendix) and anti-calbindin immunoprobed (*see* **Figure 12C**, Appendix) coronal tissue sections of the cerebellum from MAM treated animals. These stains revealed extensive hypogranulation of the cerebellum and disorganization and displacement of neurons within both the granule and Purkinje cell layers. The disorganization of granule and Purkinje cells was also evident in coronal sections stained with the nuclear stain DAPI or Fluoro-Jade B (*see* **Figure 13**, *top panels*), a high affinity fluorescent probe that has been used to detect degenerating neurons in rodents administered excitotoxins [**8**] or pesticides [**2**]. Particularly noticeable was the heavy deposition of silver stain (*green arrows*) over neurons within the molecular layer of the cerebellum (*see* **Figure 13**, *middle panels*) an indication that these cells are injured or damaged. This is supported by the identification of DNA damage (i.e., DNA strand breaks) by TUNEL labelling (*white arrows*) in neurons of the granule and molecular layers of MAM treated neurons or in DNAse I treated saline (+ Control) tissue sections (**Figure 13**, *bottom panels*). In contrast, TUNEL labelling was absent in coronal tissue sections of the cerebellum from saline treated mice.

These studies demonstrate that MAM severely disturbs the cytoarchitecture of the cerebellum by inducing severe neuronal loss of cerebellar granule cells and neurodegeneration *via* a DNA damage mechanism. MAM-induced cell death of the external granule cell layer of the cerebellum of wild type mice has recently been observed in 3-day old postnatal wild type mice administered an intraperitoneal injection of MAM [**4**]. Studies are currently underway with $MGMT^{+}$ and other DNA repair mutant mice to determine if the extent of cerebellar neuronal loss, neurodegeneration and DNA damage is exacerbated or reduced in mice with perturbed DNA repair.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that neurons are more sensitive to HN2 and MAM than comparably treated astrocytes or non-nervous tissue (i.e., fibroblasts).
- Demonstrated that cerebellar neurons from wild type mice (*in general*) are more sensitive to MAM and HN2 than neurons from other brain regions (e.g., cortex, midbrain).
- Demonstrated that neurons deficient in AAG are equally sensitive to the acute toxic effects of MAM or HN2 as wild type cells.
- Demonstrated that neurons deficient in MGMT and XPA are more sensitive than wild type cells to the acute toxic effects of MAM or HN2.
- Demonstrated that MAM and HN2-induced DNA damage (i.e., AP sites) persists in neurons, but not in fibroblasts of AAG or XPA deficient mice.
- Demonstrated that the extent of DNA damage differs for HN2 and MAM among DNA repair deficient neurons (i.e., $XPA^{-/-}$ *vs.* $AAG^{-/-}$).
- Demonstrated that the long-term survival (i.e., 2-3 weeks) of MAM and HN2 treated fibroblasts is dependent upon the DNA repair capacity of cells, evidence suggesting that similar effects may occur in toxin treated neurons.
- Demonstrated that HN2 appears to kill neurons by an apoptotic mechanism and MGMT may play an important role in influencing this cell death process.
- Demonstrated that *in vivo* MAM induces DNA damage, severe loss of wild type cerebellar granule cells and extensive disorganization of the cerebellar cytoarchitechture.

REPORTABLE OUTCOMES

1. Kisby, G.E., Wong, V., Olivas, A., Qin, X., Gerson, S.L., Samson, L., Turker, M.S. Neurons of DNA repair mutant mice are selctively vulnerable to DNA damage. Soc Neurosci Abstr (*In press*). Findings from the second and third year of studies will be presented at the SON Meeting on Nov 10-15, 2001 in San Diego, CA.

2. T. Nouspikel, S. LeDoux, G. Kisby, P. Brooks, P. Hanawalt, DNA repair in the nervous system. Neuron (In preparation, *see Appendix*).

3. Kisby, G.E., Sweatt, C,. Gilchrist, J., Zelenka, J., Vemana, S., Komma, G., Wong, V., Qin, X., Gerson, S.L., Turker, M.S. The DNA repair protein O^6-methylguanine methyltransferase (MGMT) protects neurons from methylazoxymethanol (MAM)-induced cell death. J Neurochem (In preparation, *see Appendix*).

CONCLUSIONS

Cytotoxocity Studies

The central hypothesis under study in this grant is that DNA damage is a primary mechanism of mustard-induced neuronal cell death. Our primary strategy for exploring this hypothesis is to use mouse models with perturbations in different DNA repair pathways to examine the acute and delayed neurotoxic properties of mustards (i.e., nitrogen mustard, HN2). For comparison, DNA repair mutant mice were also to be treated with methylazoxymethanol (MAM), an environmental agent that is strongly linked with a neurological disorder with features of Parkinson's disease and dementia. The selective vulnerability of neurons within the CNS is one of the key features of Parkinson's disease and related neurodegenerative disorders. Consistent with this notion, we demonstrate that neurons (*vs.* astrocytes) are selectively vulnerable to MAM and HN2 and that this vulnerablity also differs, at least *in vitro*, on a regional level (cerebellar > cortex > midbrain) for neurons. The mechanism underlying this regional and cell specific vulnerability was further examined by comparing the acute neurotoxicity of HN2 and MAM in cerebellar neurons from mice with deficits in different DNA repair pathways [i.e., direct reversal (MGMT), base-excision (AAG) and nucleotide excision (XPA)]. The objective here is that if DNA damage is a key mechanism underlying the neurotoxicity of MAM and HN2, we would expect that DNA repair-deficient neurons from the same brain region and treated in a similar manner with HN2 or MAM would exhibit differences in sensitivity. Subsequently, DNA repair-deficient neurons that exhibit an increased sensitivity to HN2 or MAM would be expected to have elevated levels of DNA damage and, possibly of certain types. In general, findings from Year 3 studies indicate that DNA repair capacity and DNA damage are important determinants of the vulnerability of neurons to the acute toxicity of both HN2 and MAM. For example, $AAG^{-/-}$ cerebellar neurons were equally vulnerable as wild type cells to both MAM and HN2 while $MGMT^{-/-}$ and $XPA^{-/-}$ neurons were more sensitive to HN2 or MAM. As proposed, these studies indicate that the acute neurotoxicity of HN2 and MAM depends upon the DNA repair capacity of neurons. This pattern of sensitivity to HN2 and MAM (24h pulse treatment) was also observed in long-term studies of $AAG^{-/-}$, $MGMT^{-/-}$ and $XPA^{-/-}$ fibroblasts. The long-term survival of $AAG^{-/-}$ fibroblasts was equal to or better than HN2 or MAM treated wild type or DNA repair deficient fibroblasts (i.e., $MGMT^{-/-}$, $XPA^{-/-}$), respectively. These long-term studies of fibroblasts from the same DNA repair-deficient mice suggest that neurons of these mice may be more prone to the delayed toxic effects of HN2 and MAM. Comparable studies are proposed in Year 4 to determine if a pulse treatment (24h) of repair-deficient neurons (i.e., $MGMT^{-/-}$, $XPA^{-/-}$) with HN2 or MAM (at non-lethal concentrations from acute studies) leads to an increased sensitivity these cells weeks later.

Findings from these studies will also be important for determining which DNA repair-deficient mouse to use for examining the *in vivo* delayed or chronic neurotoxic effects of HN2 or MAM.

However, the acute toxicity of HN2 or MAM (unlike comparably treated neurons) was noted to be essentially the same for fibroblasts from all three DNA repair-deficient animals. These findings suggest that there may be tissue specific differences for the acute toxicity of these compounds, as previously reported for $AAG^{-/-}$ cells [**6**]. An alternative explanation is that the proliferative state of a cell may also be an important determinant of the acute toxicity of HN2 and MAM. Studies are proposed in Year 4 to resolve these differences by comparing the acute toxicity of HN2 and MAM in astrocytes (a proliferative CNS cell type) and fibroblasts from the same DNA repair deficient.

DNA Damage Studies

DNA damage was also examined in HN2 and MAM treated cerebellar neurons from wild type and DNA repair-deficient mice to determine if the acute neurotoxic effects of HN2 and MAM were related to an increase in the levels and/or type of damage. Cerebellar neurons from wild type and DNA repair deficient mice were treated with HN2 and MAM and examined for the level of AP sites, strand breaks, or DNA fragmentation, but not damage to nucleobases (e.g., O^6-mG, N^7-mG, N^7-alkylG). AP sites were detected in both $AAG^{-/-}$ and $XPA^{-/-}$ cerebellar neurons treated with HN2 or MAM (at non-lethal concentrations of the toxins), but the levels were higher in toxin treated $XPA^{-/-}$ neurons. In contrast, we did not observe an increase in the levels of AP sites over that of controls in comparably treated $AAG^{-/-}$ and $XPA^{-/-}$ fibroblasts. DNA fragmentation was also examined in HN2 and MAM treated $MGMT^{-/-}$, $AAG^{-/-}$, or $XPA^{-/-}$ neurons and it only occurred in neurons treated with high concentrations of the toxins. These studies demonstrate that DNA damage preferentially accumulates in HN2 and MAM treated neurons (*vs.* non-nervous tissue) and that the extent of DNA damage (like cytotoxicity) is dependent upon the efficiency of neuronal DNA repair. These findings, however, suggest that the relationship between the extent or type of DNA damage and the acute neurotoxic effects of HN2 and MAM is less clear. One possibility is that the persistence of certain types of DNA adducts may be an important factor in determining whether DNA damaging agents induce delayed (a leading hypothesis of the grant) rather than acute neurotoxicity whereas others may induce an apoptotic cascade. For example, N^7-alkylG or N^7-mG DNA adducts are non-lethal DNA lesions, but with age they depurinate to DNA adducts which are cytotoxic and mutagenic. In support of the former, we demonstrated that low concentrations of HN2 and MAM are not acutely toxic to $MGMT^{-/-}$ or $XPA^{-/-}$ neurons and fibroblasts, but when the same cells are pulsed (24h) with the same concentrations of these toxins and examined weeks later, their viability is reduced. Moreover, neuronal loss, degeneration and DNA damage were observed in the cerebellum of wild type mice ~1 month after postnatal mice were administered MAM. Studies in Year 4 are expected to clarify these differences by examining HN2 and MAM treated DNA repair-deficient neurons for nucleobase DNA damage and for delayed toxicity following a pulse treatment (similar to that of fibroblasts) with non-lethal concentrations of the toxins.

A comparison of the short-term (i.e., acute) and long-term (i.e., delayed) survival of HN2 and MAM-treated neuronal or non-neuronal tissue (i.e., fibroblasts) in Year 3 studies has provided preliminary evidence to support our hypothesis that DNA damage is an important mechanism underlying the neurotoxicity of HN2 and MAM. Studies are now underway (Year 4) to confirm this hypothesis by comparing the viability of neuronal cultures from DNA repair deficient mice after a pulse (24h) of the toxins (similar to Year 3 fibroblast studies) or continuous exposure to HN2 or MAM for up to 2 weeks. Findings from these studies will complement the long-term survival studies conducted with fibroblasts treated with HN2 and MAM in Year 3 and resolve differences in the sensitivity of post-mitotic and

dividing cells to these toxins. DNA damage will be assessed on similarly treated neurons to determine if there is a relationship between the extent/type of DNA damage and the delayed neurotoxicity of HN2 and MAM. In parallel with these studies, DNA repair-deficient mice will be administered MAM and HN2 and examined for neuronal loss, degeneration and DNA damage as previously conducted with wild type mice in Year 3 studies. Findings from these Year 4 *in vitro* and *in vivo* studies should provide sufficient evidence to clarify the role of DNA damage in MAM- and HN2-induced neuronal cell death.

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APPENDIX

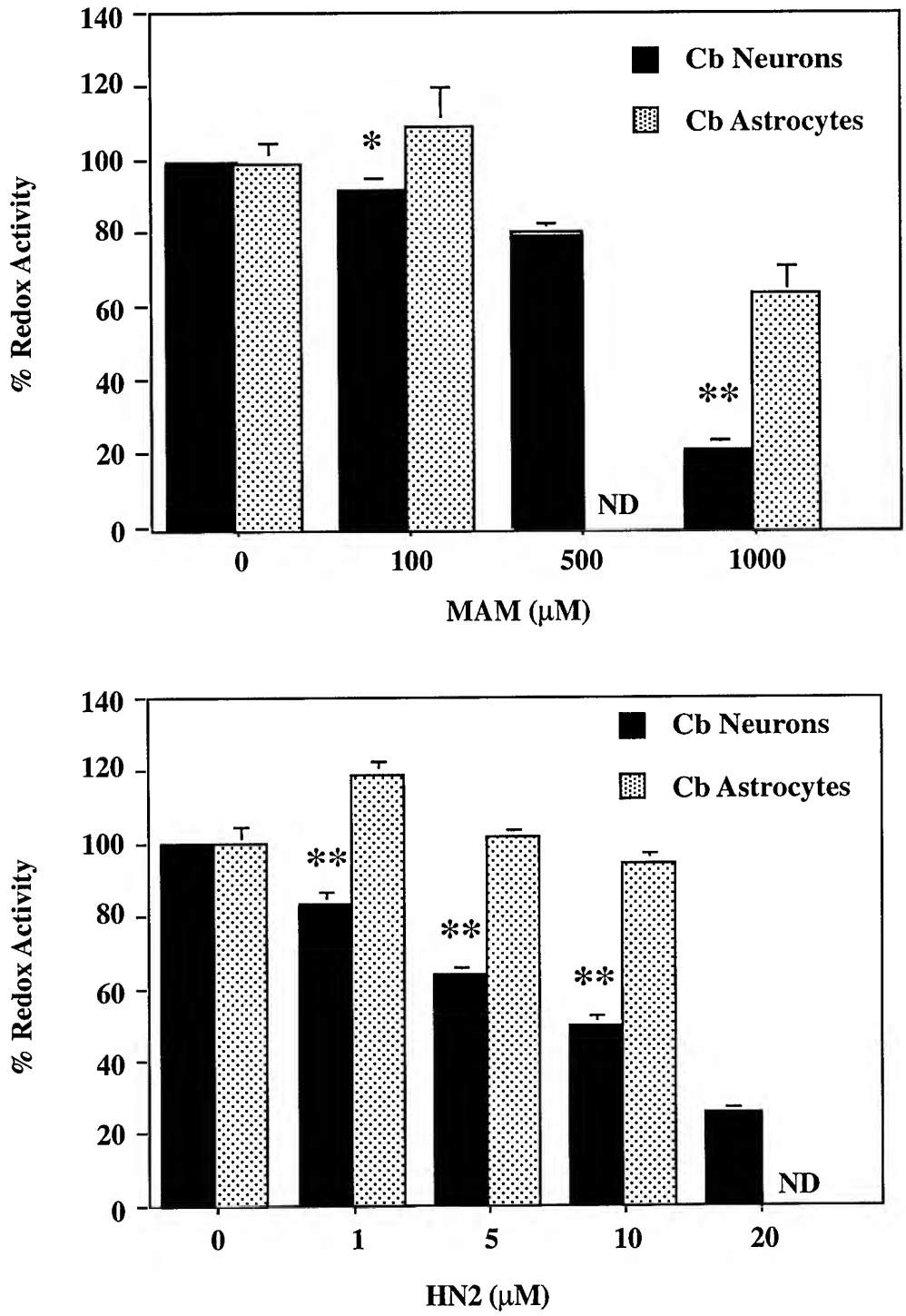

Figure 1. Comparative Toxicity of MAM and HN2 Among Different CNS Cell Types. Primary neuronal and astrocyte cell cultures were prepared from the cerebellum of 6-7 day old C57BL/6 (wild type) mice and maintained for 7 or 14 days (respectively) before treatment for 24h with 100 μM-1000 μM methylazoxymethanol (MAM) or 1 μM-20 μM nitrogen mustard (HN2). Toxin treated cultures were examined for cell viability by incubating the cells for 4h with the redox fluorescent dye Alamar Blue™. Values represent the mean ± SEM of 3-6 separate experiments (*n= 4*). Significantly different from MAM treated astrocytes (** $p < 0.05$*, *** $p < 0.001$*, ANOVA). ND= not determined.

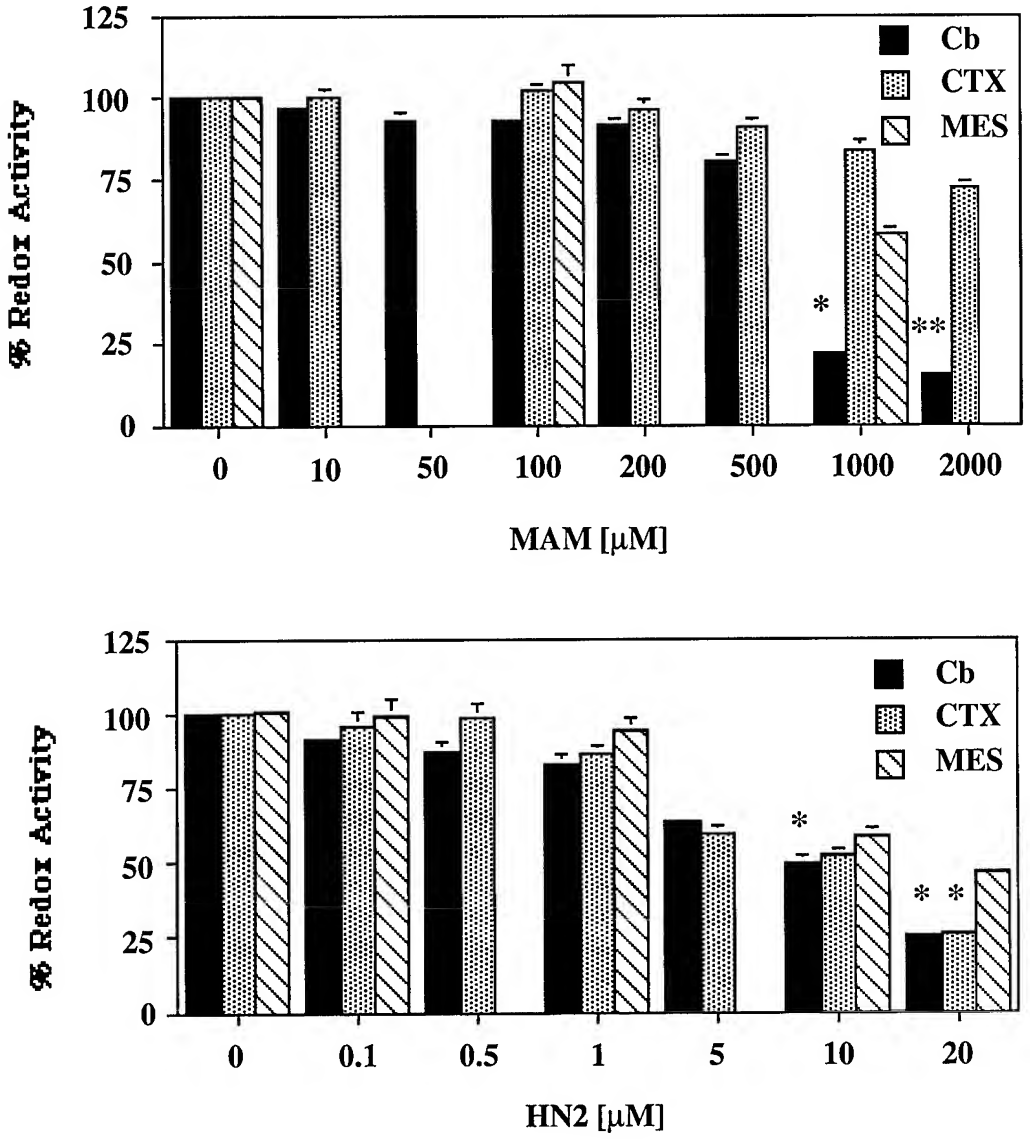

Figure 2. Comparative Toxicity of MAM and HN2 Among Neurons from Different Brain Regions. Primary neuronal cell cultures were prepared from the cerebellum (Cb), cerebral cortex (CTX) or midbrain (MES) of C57BL/6 (wild type) mice, the cultures maintained for 7d before treatment for 24h with 10 μM-2000 μM methylazoxymethanol (MAM) or 0.1 μM-20 μM nitrogen mustard (HN2). The cultures were incubated with Alamar Blue™ for 4h and examined for fluorescence. Values represent the mean % redox activity of controls ± SEM of 3-6 separate experiments (*n= 4*). Significantly different from controls (** p <0.001*, ANOVA).

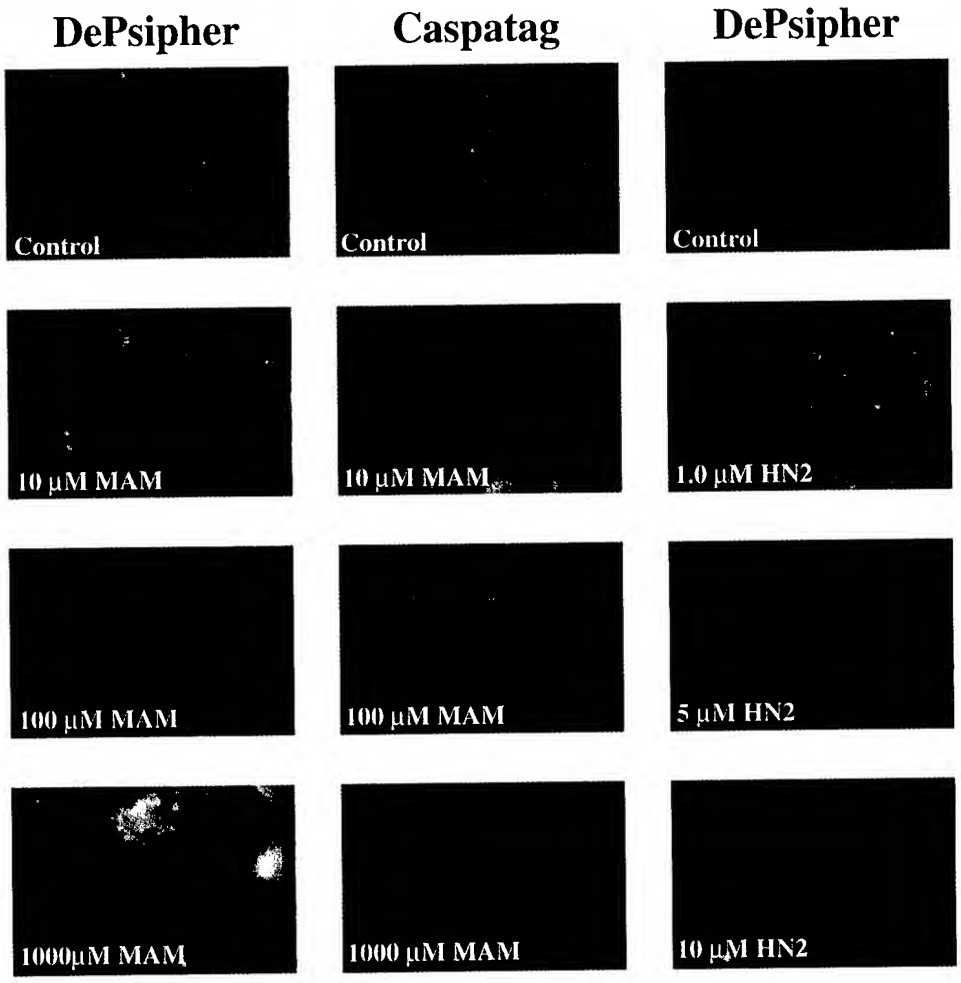

Figure 3. Markers of Apoptosis in MAM and HN2 Treated Wild Type (C57 BL/6) Neurons. Representative fluorescent micrographs from primary cerebellar neurons treated with various concentrations of MAM (10 μM-1000 μM) or HN2 (1.0 μM-10 μM) for 24h, and the cultures examined for the integrity of the mitochondrial membrane potential using the fluorescent dye DePsipher™ (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethybenzimidazolyl carbocyanine iodide or JC-1, Trevigen Inc.) or the activation of cellular caspases using the caspase inhibitor CaspaTag™ (FAM-VAD-FMK, Intergen, Inc.). Fluorescent orange-red aggregates accumulate in mitochondria with an intact membrane potential while the green monomer accumulates in cells with a perturbed mitochondrial membrane potential. Cells undergoing apoptosis fluoresce intense green after incubation with the caspase inhibitor FAM-VAD-FMK. Note the marked effect of HN2 at low concentrations (5.0 μM or greater) on the mitochondrial membrane potential when compared with MAM treated neurons (compare left and right panels). Note also the lack of effect by MAM to activate neuronal caspases.

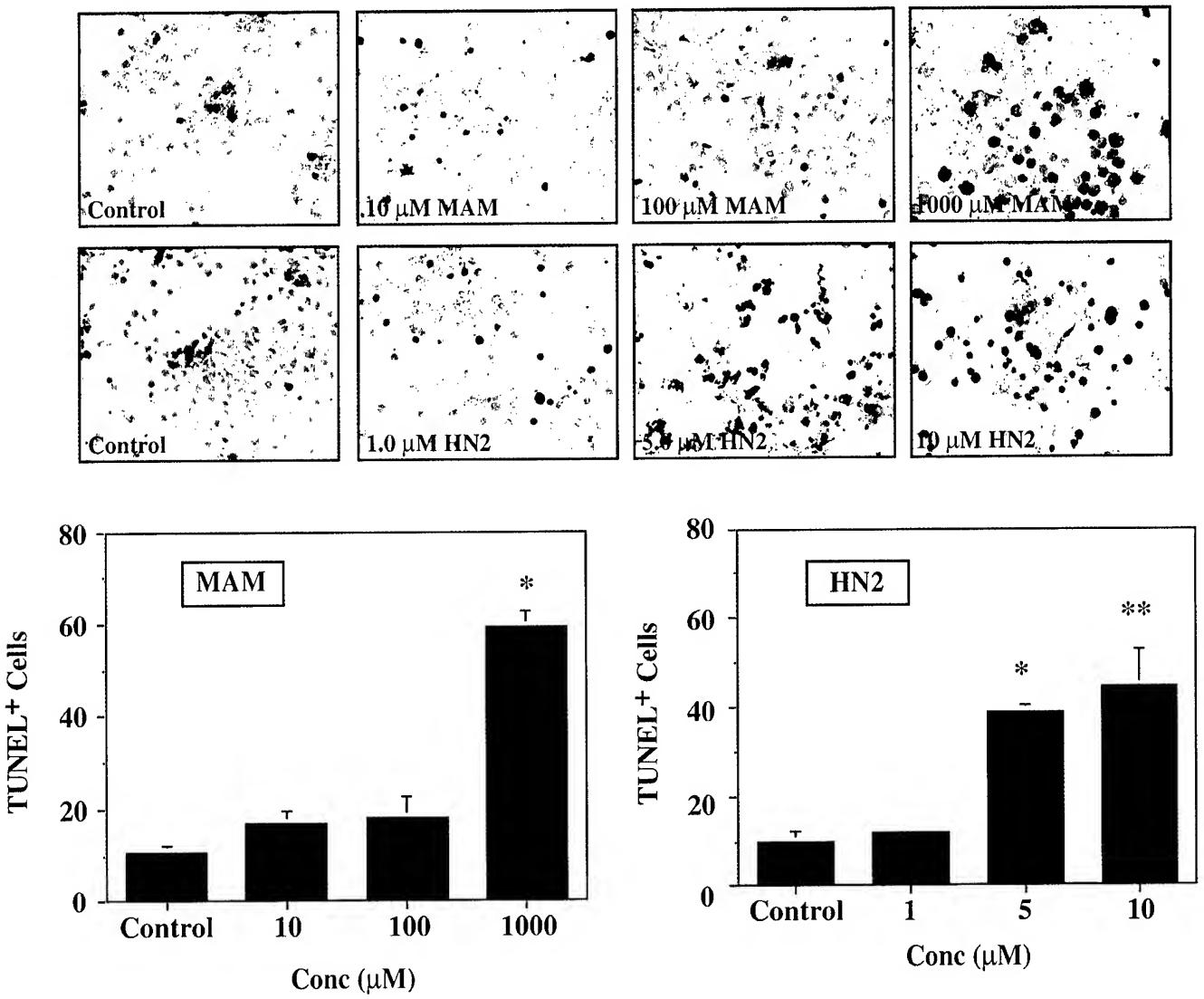

Figure 4. ***In situ*** **DNA Damage of Wild Type (C57BL/6) Neurons by MAM and HN2.** Representative light micrographs of primary cerebellar neurons that were treated for 24h with various concentrations of MAM (10 μM-1000 μM) or HN2 (1.0 μM-10 μM) and examined for DNA damage using the NeuroTacs™ kit (Trevigen, Inc.). The NeuroTacs™ kit detects DNA strand breaks in cells by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique. After toxin treatment, cells (on chamber slides) were fixed with 4% buffered paraformaldehyde and the incorporation of biotinylated nucleotides determined by incubating the sections with NovaRed™ (Vector Labs, Inc) according to the manufacturer's protocols. The slides were washed, counterstained for 30 min with methyl green (Vector Labs, Inc.), mounted and the cells examined by light microscopy on a Zeiss Axioskop 2 microscope with digital imaging software (i.e., AxioVision 3.0). For quantitative studies, 5 random fields (~1000 cells/field of only cells with prominent nuclei) were counted and the values expressed as the mean ± SEM of immunopositive cells ($TUNEL^+$). Note the extensive TUNEL labelling of neurons treated with 5.0 μM and 10 μM HN2 while significant DNA damage was only observed at the highest concentration of MAM. Significantly different from control (*$p<0.01$, **$p<0.001$, ANOVA).

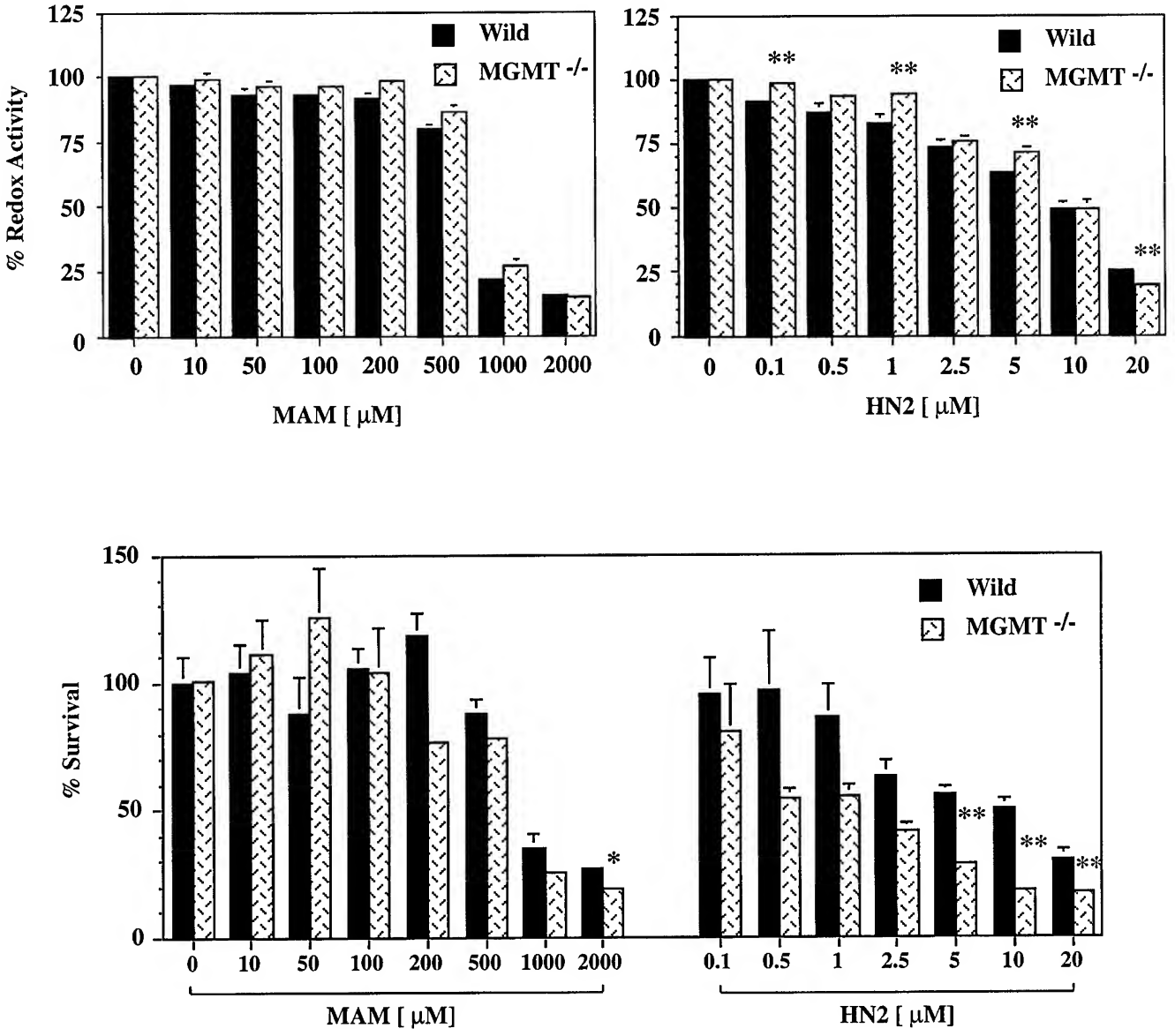

Figure 5. Viability of MGMT-deficient (MGMT$^{-/-}$) Cerebellar Neurons Treated with MAM or HN2. Mouse cerebellar granule cell cultures were treated with various concentrations of MAM (10 μM-2000 μM) or HN2 (0.1 μM-20 μM) for 24h, the cultures incubated with Alamar Blue™ for 4h and examined for fluorescence (*top figures*). After 4h, the culture media was removed and replaced with culture media containing 0.26 μM calcein-AM and 3.0 μM propidium iodide, the cells incubated for 10 min, washed with control media, and cell survival examined on a fluorescence microplate reader (GeminiXS™, Molecular Devices) with well-scan capabilities (*bottom figures*). Values represent the mean % redox activity (*top*) or % surviving cells (*botttom*) of controls ± SEM (*n= 6/group x 2-5 experiments*). Significantly different from toxin treated wild-type cells (* $p < 0.05$, ** $p < 0.01$, ANOVA).

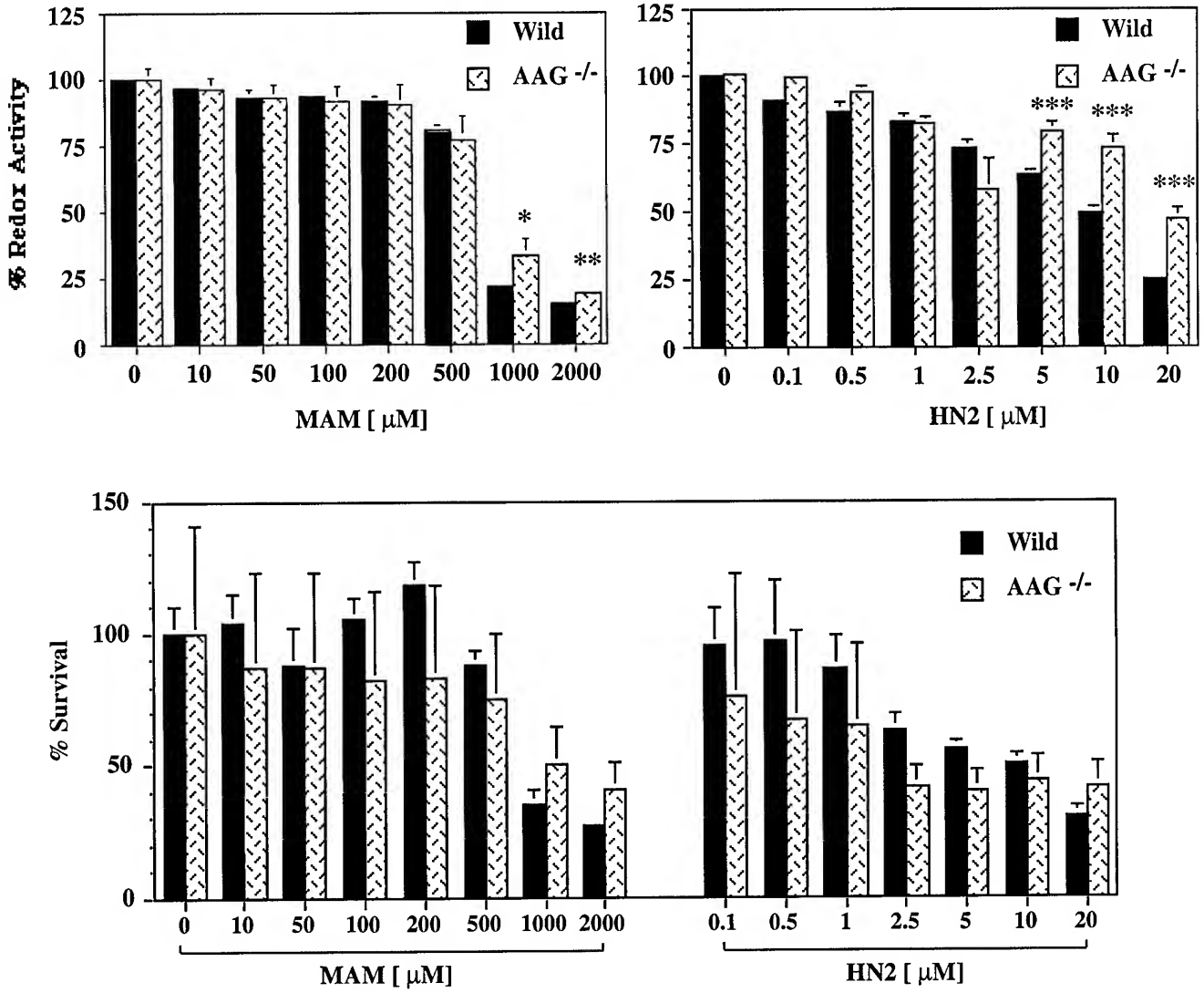

Figure 6. Viability of AAG-deficient ($AAG^{-/-}$) Cerebellar Neurons Treated with MAM or HN2. Mouse cerebellar granule cell cultures were treated with various concentrations of MAM (10 μM-2000 μM) or HN2 (0.1 μM-20 μM) for 24h, the cultures incubated with 10% Alamar Blue™ for 4h and examined for fluorescence (*top figures*). After 4h, the culture media was removed and replaced with culture media containing 0.26 μM calcein-AM and 3.0 μM propidium iodide, the cells incubated for 10 min, washed with control media, and cell survival examined on a fluorescence microplate reader (*bottom figures*). Values represent the mean % redox activity (*top*) or % surviving cells (*botttom*) of controls ± SEM (*n= 6/group x 2-5 experiments*). Significantly different from toxin treated wild-type cells (* $p < 0.05$, ** $p< 0.01$, *** $p <0.01$, ANOVA).

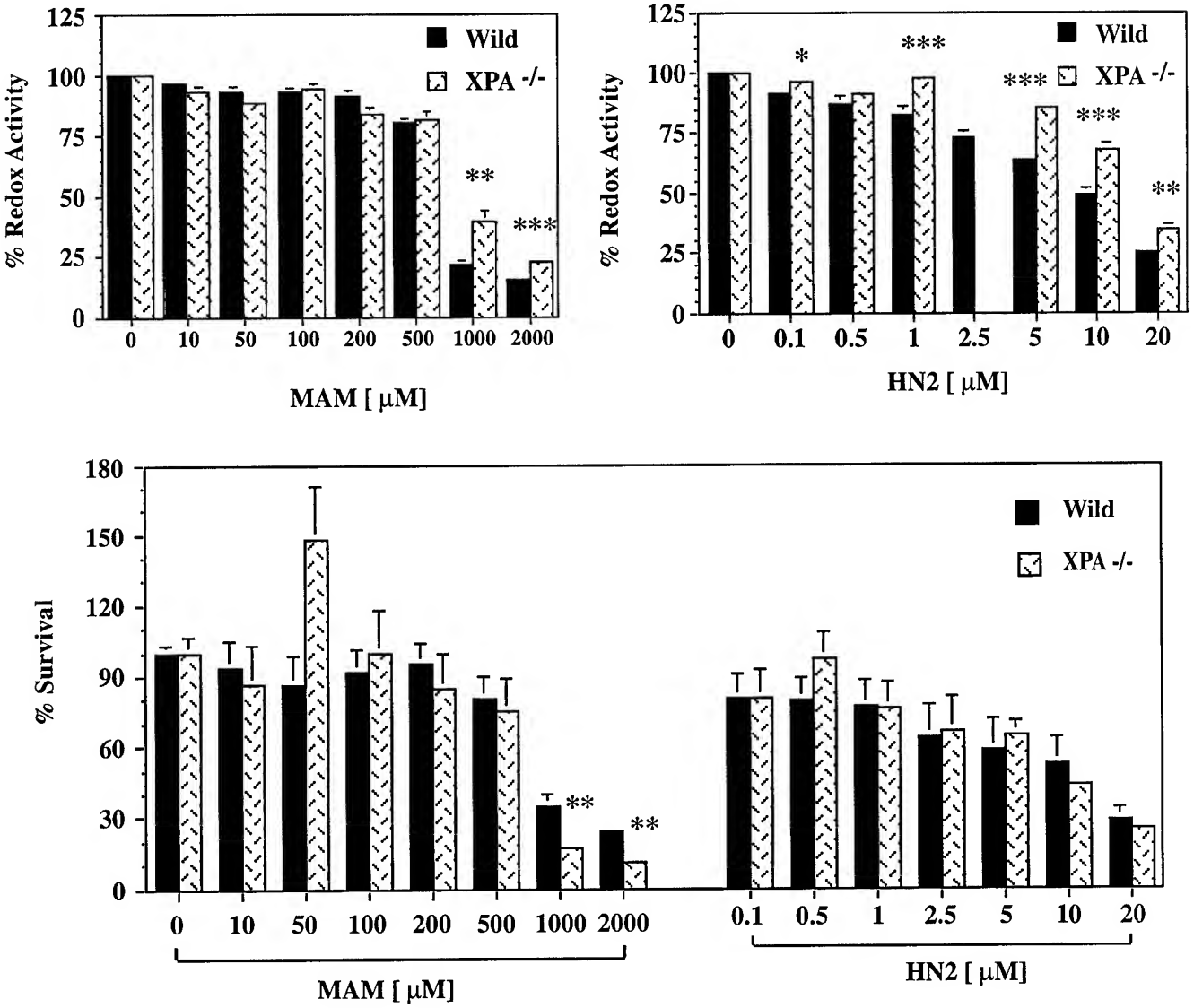

Figure 7. Viability of XPA-deficient ($XPA^{-/-}$) Cerebellar Neurons Treated with MAM or HN2. Mouse cerebellar granule cell neuronal cultures were treated with various concentrations of HN2 (0.1 μM - 10 μM) or MAM (50 μM - 1000 μM) for 24h, the cultures incubated with Alamar Blue™ for 4h and examined for fluorescence (*top figures*). After 4h, the culture media was removed and replaced with culture media containing 0.26 μM calcein-AM and 3.0 μM propidium iodide, the cells incubated for 30 min, washed with control media, and cell survival examined on a fluorescence microplate reader with well-scan capabilities (*bottom figures*). Values represent the mean % redox activity (*top*) or % surviving cells (*botttom*) of controls ± SEM (*n= 6/group x 2-5 experiments*). Significantly different from toxin treated wild-type cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$, ANOVA).

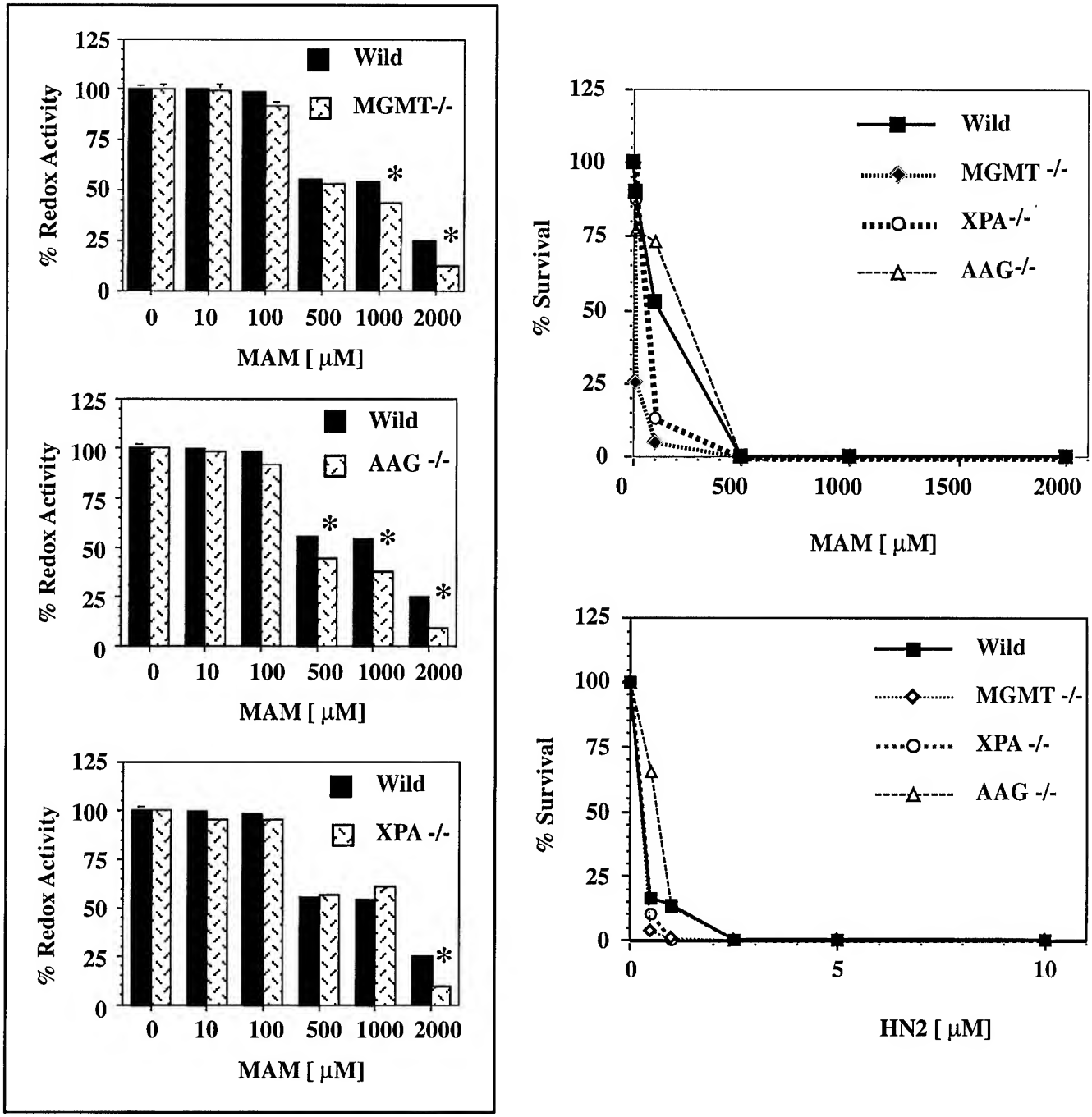

Figure 8. Viability of Wild-Type (C57BL6), MGMT$^{-/-}$, AAG$^{-/-}$, and XPA$^{-/-}$ mouse skin fibroblasts treated with MAM. Skin fibroblast cell lines were developed from the ears of wild type, MGMT$^{-/-}$, AAG$^{-/-}$, and XPA$^{-/-}$ mice, the cells seeded at a density of 30,000 cells/well (short-term studies, *left*) or 4000 cells/dish (long-term studies, *right*). The cultures were treated for 24h with various concentrations of HN2 (1.0 μM - 20 μM) or MAM (100 μM - 1000 μM) and the cells either incubated with Alamar Blue™ for 4h and examined for viability (*short-term studies*) or the toxin media removed, replaced with control culture media and the cloning efficiency of 2-3 week old cultures determined on cresyl violet stained culture dishes (*long-term studies*). Values represent the mean ± SEM (*n= 2 experiments*). Significantly different from wild-type cells (**p <0.001*, ANOVA).

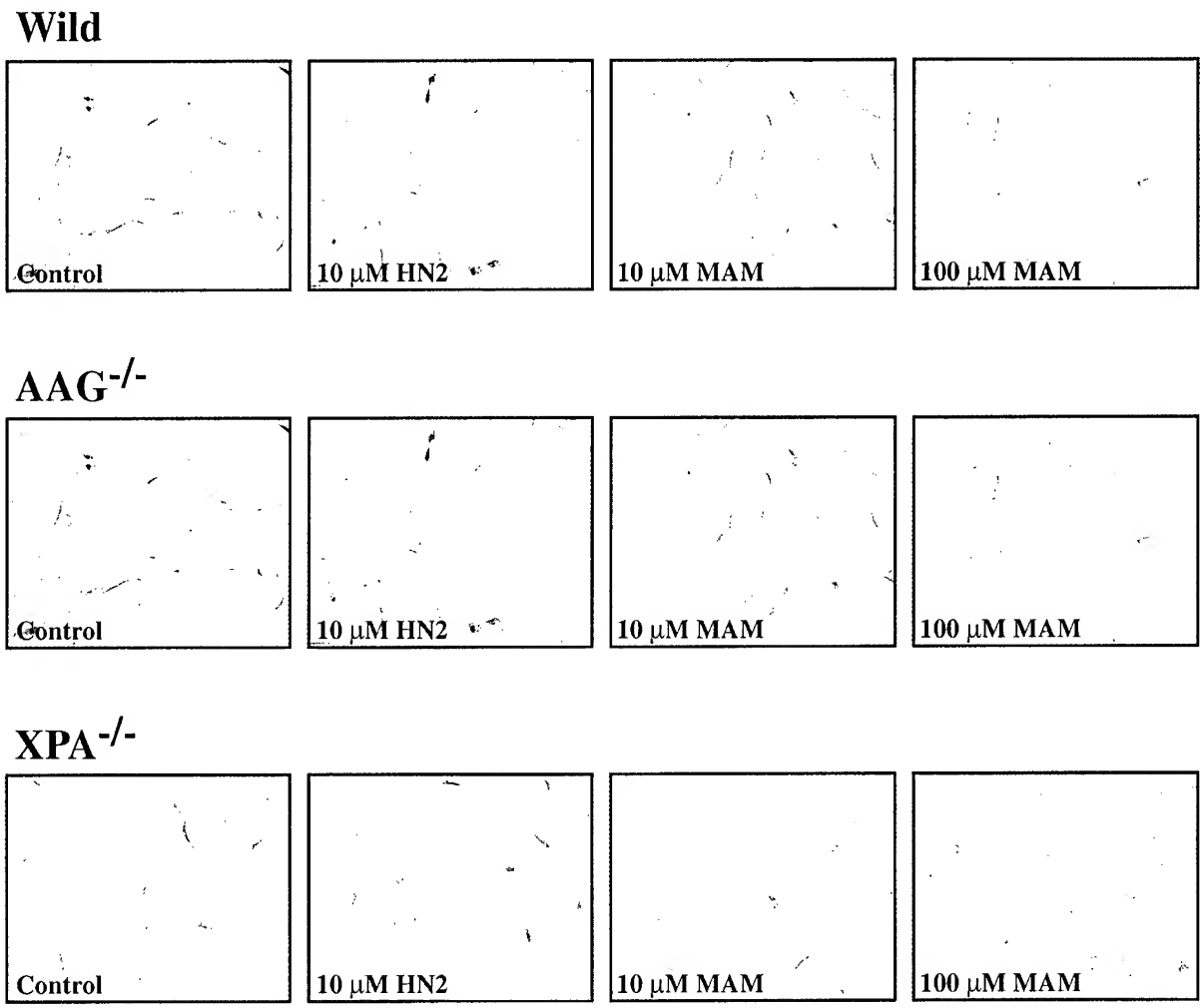

Figure 9. *In situ* DNA Damage of Wild-Type, AAG$^{-/-}$, and XPA$^{-/-}$ Mouse Fibroblasts Treated with HN2 and MAM. Skin fibroblast cell lines from the ears of wild type (C57BL/6), AAG$^{-/-}$, and XPA$^{-/-}$ mice were seeded at a density of 2000 cells/well (8-chamber slides) and the cultures allowed to grow until 80% confluency before they were treated for 24h with control culture media or culture media containing various concentrations of HN2 (0.1 μM, 1.0 μM, 10 μM) or MAM (10 μM, 100 μM, 1000 μM). Representative light micrographs of toxin treated skin fibroblasts that were examined for DNA damage using the NeuroTacs kit (Trevigen, Inc.), a TUNEL technique (*for details* see **Figure 4**). After toxin treatment, cells (on chamber slides) were fixed with 4% buffered paraformaldehyde and the incorporation of biotinylated nucleotides determined by incubating the cultures with NovaRed™ (Vector Labs, Inc) according to the manufacurer's protocols. The slides were washed with buffer, counterstained for 30 min with methyl green (Vector Labs, Inc.), mounted and the cells examined by light microscopy on a Zeiss Axioskop 2 microscope with digital imaging software (i.e., AxioVision 3.0). Note the lack of TUNEL labelling of wild type or DNA repair deficient fibroblasts treated either with HN2 or MAM.

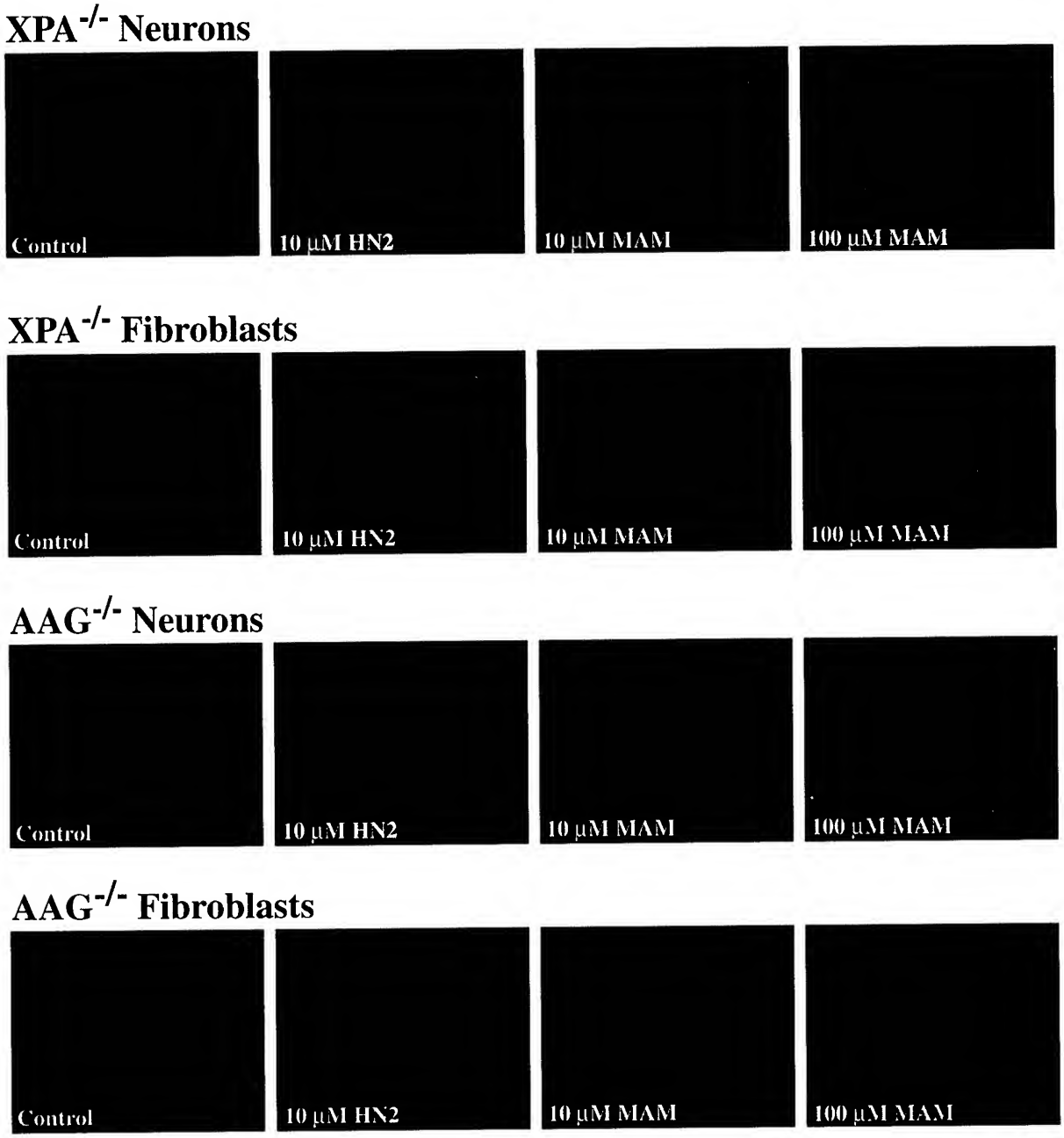

Figure 10. *In situ* Detection of AP Sites in $AAG^{-/-}$ and $XPA^{-/-}$ Mouse Skin Fibroblasts and Cerebellar Neurons Treated with HN2 or MAM. Skin fibroblast cell lines from the ears or primary neurons from the cerebellum of $AAG^{-/-}$, and $XPA^{-/-}$ mice were treated for 24h with various concentrations of HN2 (1.0 μM-10 μM) or MAM (100 μM - 1000 μM) and examined for the extent of apurinic (AP) sites by incubating live cells for 30 min with 1.0 mM aldehyde reactive probe (ARP, gift from Dr. W. Kow, Emory University, Atlanta, GA). After ARP treatment, the cells were extensively washed, fixed with methanol (aldehyde-free), treated briefly with RNAse A, and labeled with CY3 conjugated streptavidin (1:100). The slides were washed with buffer, coverslipped in low fluorescent media mounting media (glycerol with *p*-phenylaminediamine), and examined by light microscopy on a Zeiss Axioskop 2 microscope with digital imaging software (i.e., AxioVision 3.0). Note the lack of TUNEL labelling of wild type or DNA repair deficient fibroblasts treated either with HN2 or MAM.

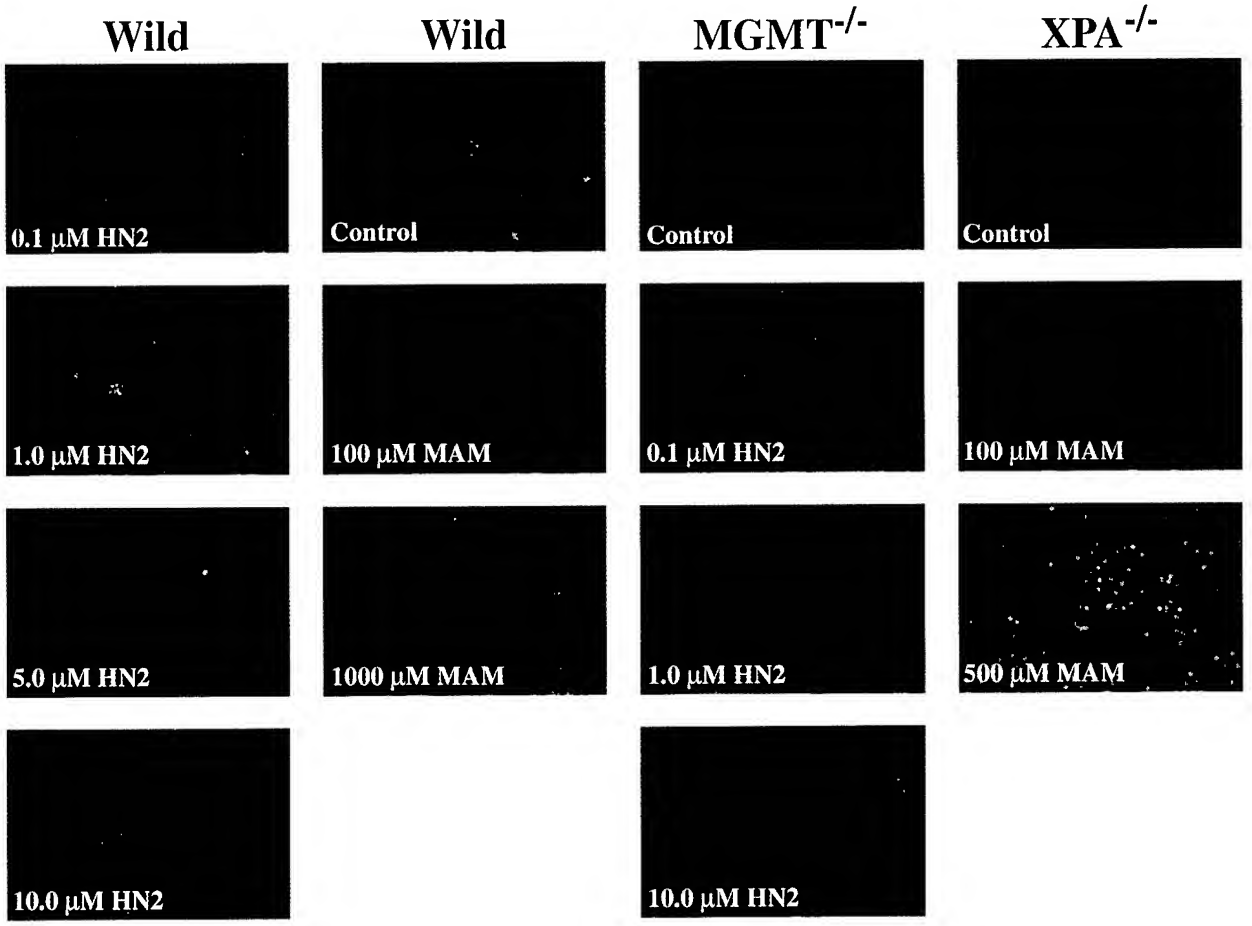

Figure 11. *In situ* Detection of DNA Fragmentation in Wild Type, MGMT$^{-/-}$ and XPA$^{-/-}$ cerebellar neurons treated with HN2 or MAM. Primary neurons from the cerebellum of C57Bl/6 (wild type), MGMT$^{-/-}$, and XPA$^{-/-}$ mice were treated for 24h with various concentrations of HN2 (1.0 μM-10 μM) or MAM (100 μM - 1000 μM) and examined for the extent of DNA fragmentation (apoptotic bodies) by incubating the cells for 30 min with Hoescht 33342 (HO). After treatment with HO, the cells were washed with buffer, coverslipped in low fluorescent mounting media (glycerol with *p*-phenylaminediamine), and examined by epifluoresence microscopy on a Zeiss Axioskop 2 microscope with digital imaging software (i.e., AxioVision 3.0).

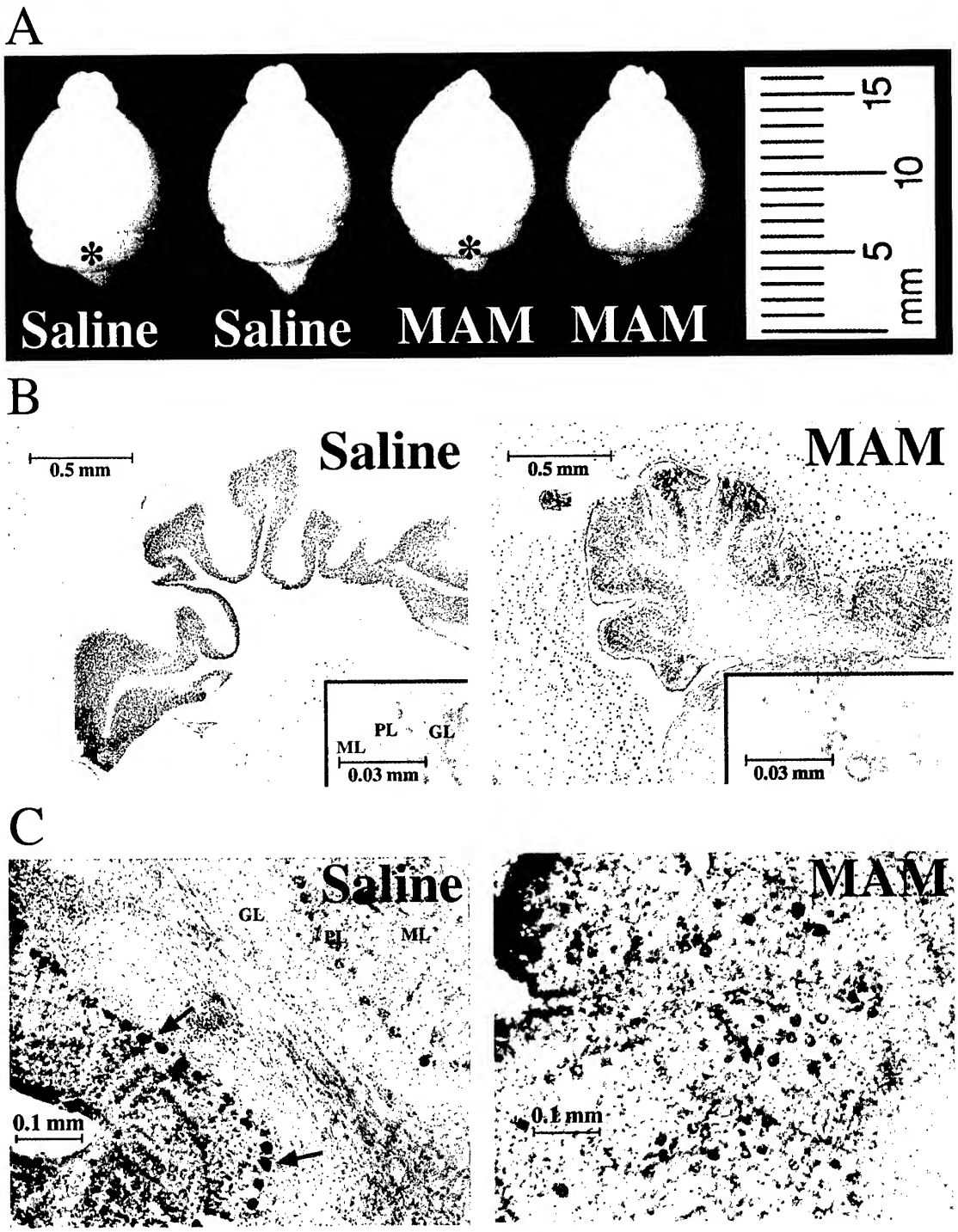

Figure 12. Morphology of the Cerebellum from Postnatal C57BL/6 Mice treated with MAM. Gross observation of the brain of MAM treated mice and light micrographs of representative areas from the cerebellum of 21 day-old pups treated at postnatal day 1-2 with saline (control) or MAM (30 mg/kg, s.c.) (*bottom panels*). Note the smaller cerebellum (*stars*) of MAM *vs.* saline treated mice (**A**), but similar size of the respective cerebral hemispheres. At the light microscopic level (**B**), smaller cerebellar folia (F), thinner cerebellar cortex and disorganization of neurons in the granule (GL), Purkinje (PL) and molecular (ML) layers (*inset* in **B**) were observed. CaBP was particularly useful for visualizing the disorganization and abnormal appearance of Purkinje cells in MAM treated animals (**C**).

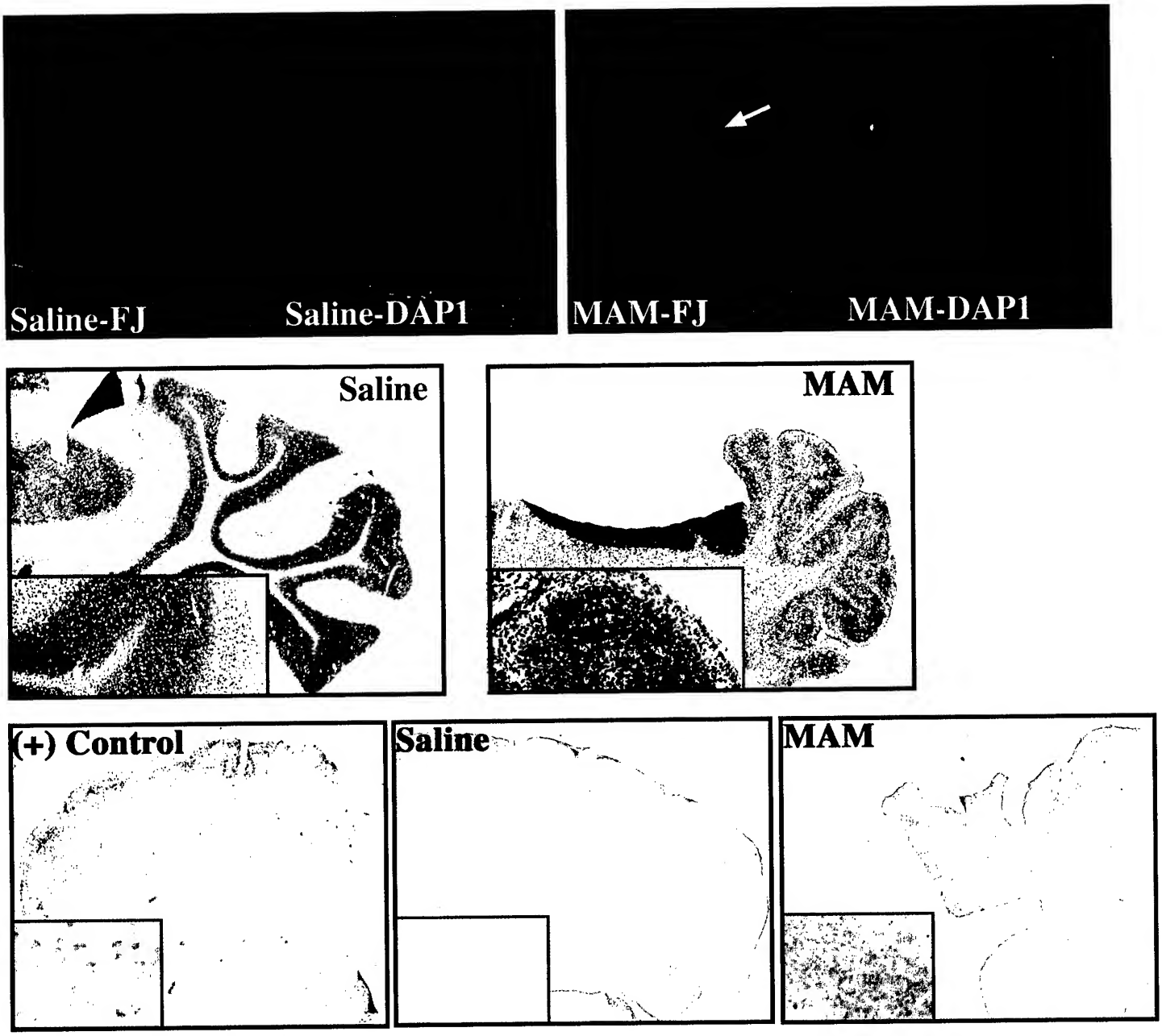

Figure 13. Neuropathology and DNA Damage in the Cerebellum of Postnatal C57BL/6 Mice Treated with MAM. Fluorescent and light micrographs of representative areas from coronal sections (25 μm) of the cerebellum of 21 day-old pups treated at postnatal day 1-2 with saline (SAL) or MAM (30 mg/kg, s.c.). Neuronal degeneration was determined by examining tissue sections incubated with the anionic fluorescein dye Fluoro-Jade B™ (FJ, Histo-Chem Inc) and the nuclear stain DAPI or silver stain (NeuroSilver™, FD Technologies) according to the manufacturer's protocols. Note the intense fluorescence of some neurons in the granule cell layer (arrow, *top panels)* and the intense precipitate over silver stained cerebellar neurons (green arrows, *middle panels*) in MAM treated animals. Numerous cells labelled with terminal deoxynucleotidyl transferase (TdT) and dUTP (TUNEL) were also observed in the granule and molecular cell layers of MAM treated animals and in tissue sections of saline treated animals incubated with DNAse I (+ Control) (*bottom panels*).

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DRAFT VERSION

THE DNA REPAIR PROTEIN O^6-METHYLGUANINE METHYLTRANSFERASE (MGMT) PROTECTS NEURONS FROM METHYLAZOXYMETHANOL (MAM)-INDUCED CELL DEATH

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ABSTRACT

Methylazoxymethanol (MAM), a developmental neurotoxin and an etiological candidate for the prototypical neurodegenerative disorder western Pacific ALS/PDC, is proposed to target neurons *via* DNA damage, but the mechanism is poorly understood. The relationship between MAM-induced DNA damage (i.e., O^6-methylguanine, O^6-mG) and neurotoxicity was examined in mature mouse cortical explants and primary rat cortical astrocytes and cerebellar granule cell cultures treated with MAM in the presence or absence of the O^6-methylguanine methyltransferase (MGMT) inhibitor O^6-benzylguanine (BG). O^6-mG was detected in explant and neuronal cultures treated for 3 days with 100 μM MAM, but the levels were ~2x higher in explants pre-treated with 5.0 μM BG. MGMT levels were also reduced in explants and astrocytes treated with the inhibitor. For comparison, cerebellar neurons from transgenic mice that overexpress human O^6-methylguanine methyltransferase ($MGMT^+$) were treated with MAM and the alkylating agent nitrogen mustard (HN2) that forms DNA adducts not repaired by MGMT to determine if this DNA repair protein protects neurons from genotoxins. Cultures of cerebellar neurons from wild type and $MGMT^+$ mice were treated for 24h with various concentrations of MAM (10 μM-1000 μM) or HN2 (0.1 μM, 1.0 μM) and the viability determined by examining the cultures for cell density, membrane integrity and mitochondrial function. Cell densities and mitochondrial function were significantly higher in $MGMT^+$ neurons treated with 10 μM, 50 μM, and 100 μM MAM than comparably treated wild type cells. In contrast, cell densities for wild type and $MGMT^+$ were similar for cerebellar neurons treated with 0.1 μM and 1.0 μM HN2. Loss of membrane integrity (LDH leakage) was also significantly higher in control and MAM treated wild type cells than comparably treated $MGMT^+$ neurons. The insensitivity of wild type or $MGMT^+$ astrocytes to either MAM or HN2-induced toxicity suggests that these CNS cell types possess efficient mechanisms for repairing different types of DNA damage. Taken together, these findings suggest that O^6-methylguanine DNA adducts play an important role in MAM-induced neuronal cell death and that DNA repair protects neurons

from insult by genotoxins. These findings are consistent with MAM selectively targeting neurons *in vivo* through a mechanism involving DNA damage.

INTRODUCTION

For the past several years, we have been engaged in a systematic study to understand the etiology and pathogenesis of a prototypical neurodegenerative disorder in the western Pacific (Guam, Kii Peninsula of Japan, and Irian Jaya, New Guinea) that has features characteristic of amyotrophic lateral sclerosis (ALS), Parkinson's disease and an Alzheimer-like dementia (PD). Epidemiological studies indicate that individuals develop clinical features of this disorder many years after heavy exposure to raw or incompletely detoxified seed of the cycad plant (*Cycas* spp.) (Spencer, P. S. *et al.*, 1987;Spencer, P. S. *et al.*, 1990;Stone, R., 1994). This hypothesis is further supported by the extraordinarily high correlation between the cycasin content of cycad flour prepared on Guam and the age-adjusted incidence of motor neuron disease (Zhang, Z. X. *et al.*, 1996). Consistent with this hypothesis, cycad and/or its principal toxin, cycasin are reported to induce progressive (but an incompletely described) neuromuscular disease in primates and ruminants (Dastur, D. K., 1964;Shiraki, H. and Yase, Y., 1975;Williams, G. M. and Weisburger, J. H., 1986;Dastur, D. K. *et al.*, 1990). Cycasin is metabolized by plant and animal tissues to the active metabolite methylazoxymethanol (MAM), a potent DNA alkylating agent with mutagenic, carcinogenic, teratogenic and neurotoxic properties (Morgan, R. W. and Hoffmann, G. R., 1983;Kisby, G. E. *et al.*, 1999). Rodents treated *in utero* with cycasin or MAM acetate within days of birth show strikingly abnormal development of the cerebellum associated with partial destruction of the external germinal layer and ectopic and multinucleated cells (Hirono, I. and Shibuya, C., 1967;Jones, M. M. *et al.*, 1972;Lovell, K. L. and Jones, M. Z., 1980), pathological features also found in the cerebellum of individuals with ALS/PDC (Shiraki, H. and Yase, Y., 1975). Rodents treated with MAM or cycasin develop neurobehavioral changes consisting of

motor dysfunction or learning and memory impairment that declined with age (Mohammed, A. K. *et al.*, 1986;Archer, T. *et al.*, 1988;Lee, M. H. and Rabe, A., 1992). Therefore, MAM is an attractive etiological candidate for ALS/PDC (Spencer, P. S. *et al.*, 1991;Spencer, P. S. and Kisby, G. E., 1992) because the genotoxin induces neuropathological and neurobehavioral features in rodents that resemble the neurological disorder. These observations, coupled with the genotoxin properties of cycasin and its aglycone metabolite methylazoxymethanol (MAM), suggest that the environmental genotoxin is a leading candidate for the proposed environmental trigger ('slow toxin') of western Pacific ALS/PDC (Kisby, G. E. *et al.*, 1992).

The mechanism of cycasin/MAM induced neuronal cell death is poorly understood, but the prevailing hypothesis is that these genotoxins target proliferating neurons by damaging DNA, though post-mitotic neurons are also affected (Johnston, M. V. and Coyle, J. T., 1979). Although N^7-methylguanine is the predominant DNA adduct, MAM produces significant amounts of other DNA adducts (notably O^6-methylguanine, 8-methylguanine) when purified DNA (Kisby, G. E. *et al.*, 1993;Kisby, G. E. *et al.*, 1995) or cultured brain tissue (Kisby, G. E. *et al.*, 1993;Kisby, G. E. *et al.*, 1994;Kisby, G. E. *et al.*, 1995) are treated with the genotoxin. N^7-methylguanine DNA adducts have been detected in fetal (Nagata, Y. and Matsumoto, H., 1969;Matsumoto, H. *et al.*, 1972), postnatal (Matsumoto, H. *et al.*, 1972;Kisby, G. E. *et al.*, 1999) and, presumably, adult (Fischer, M. H. *et al.*, 1972;Fischer, M. H. *et al.*, 1973) brain tissue of rats treated prenatally with MAM. The toxic properties of MAM are reportedly linked to its ability to form O^6-methylguanine and N^7-methylguanine DNA adducts (Matsumoto, H. *et al.*, 1972;Matsumoto, H., 1985), but N^7-alkylguanine DNA adducts are non-lethal lesions to cells (Park, J.-W. and Ames, B. N., 1988). The N^7-alkylguanine DNA adduct is repaired by the base-excision DNA repair protein 3-methylpurine DNA glycosylase (MPG, also termed alkylguanine DNA glycosylase, AAG) while O^6-mdG is repaired by O^6-methylguanine methyltransferase (MGMT) (Mattes, W. B. *et al.*, 1996;Matijasevic, Z. *et al.*, 1996). Cells that lack MGMT are especially sensitive to alkylation-induced O^6-mdG DNA adducts (Shiraishi, A. *et al.*, 2000)

while cells that overexpress MGMT are reportedly protected from the mutagenic, carcinogenic and cytotoxic properties of various alkylating agents (Gerson, S. L. *et al.*, 1994). Pharmacological manipulation of cellular MGMT levels by the MGMT inhibitor O^6-benzylguanine (BG) (Dolan, M. E. *et al.*, 1985;Dolan, M. E. *et al.*, 1990) or gene targeting strategies (Gerson, S. L. *et al.*, 1994;Shiraishi, A. *et al.*, 2000) are current techniques used to examine the sensitivity of tissues or cells to alkylating agents. The present studies use both techniques to explore the relationship between MAM-induced DNA alkylation (i.e., O^6-mdG) and neuronal cell death by examining DNA adduct levels and cytotoxicity in neural tissue treated with BG and MAM and the sensitivity of neuronal cultures from wild type and MGMT-overexpressing mice ($MGMT^+$) to MAM. For comparison, the sulfur mustard analogue mechlorethamine (nitrogen mustard or HN2), a related alkylating agent that is also a neurotoxin, but produces predominantly N^7-alkylguanine DNA adducts and cross-links (Kisby, G. E. *et al.*, 1999;Kisby, G. E. *et al.*, 1999;Esclaire, F. *et al.*, 1999), was also examined in neuronal cultures from MGMT-overexpressing ($MGMT^+$) mice. Nitrogen mustard served as a negative control for alkylation-induced cytototoxicity in $MGMT^+$ cells because HN2 does not produce O^6-alkylguanine DNA adducts (Osborne, M. R. *et al.*, 1995;Lawley, P. D. and Phillips, D. H., 1996;Lawley, P. D. and Phillips, D. H., 1996), while MAM does (Eizirik, D. L. and Kisby, G. E., 1995;Esclaire, F. *et al.*, 1999;Esclaire, F. *et al.*, 1999). Findings from these studies demonstrate that MGMT protects neurons from MAM-induced toxicity suggesting that O^6-mdG plays an important role in genotoxin-induced neuronal cell death. Elucidating the molecular mechanism(s) underlying the action of cycasin or its active metabolite MAM on the nervous system could provide valuable new information about the etiology of western Pacific ALS/PDC and possibly other progressive age-related neurodegenerative disorders.

METHODS

Mutant Mice

MGMT transgenic ($MGMT^+$) mice were generated in C57BL/6 x SJL mice using a chimeric gene construct consisting of the chicken β-actin promoter, the human MGMT cDNA, the poly A region from bovine growth hormone and the locus control region from the human CD2 gene as previously described by Dumenco *et al.* (Dumenco, L. L. *et al.*, 1993). The brains of 6-8 day old $MGMT^+$ and isogenic wild-type littermates were placed in Hibernate/B27 cell culture media (GibcoBRL) and sent overnight (XQ) on wet ice to our laboratory for the preparation of cerebellar and astrocyte cell cultures.

Mouse Cortical Explants

Explants prepared from the motor cortex of 1-2 day old C57BL/6 mice were placed onto collagen-coated tissue culture plates and maintained for 1-2 weeks *in vitro* as previously described by Kisby *et al.* (Kisby, G. E. *et al.*, 1992). Mature cortical cultures were treated for 5 days with control media or media supplemented with 100 μM MAM, 5.0 μM O^6-benzylguanine (BG; provided by Dr. R. Moschel, NCI) or 100 μM MAM + 5.0 μM O^6-benzylguanine (MAM + BG) and the tissue examined for viability or processed for O^6-methylguanine DNA adducts and DNA repair.

Rat Neuronal and Astrocyte Cell Cultures

Primary granule cell or cortical astrocyte cell cultures were prepared from the cerebella of 8-day old or the cerebra of 1-2 day old neonatal Sprague Dawley rats by dissociating the tissue in trypsin as previously described (Kisby, G. E. and Acosta, D., 1987). Astrocytes were diluted with MEM (with 10% fetal calf serum) and plated at a density of 0.4 x 10^6 cells/60 mm dish. Cerebellar neurons were diluted with plating media (MEM with 10% FCS, 10% HS, 600 mg/ml glucose and 30 μg/ml insulin) seeded at a density of 2-3 x 10^6 cells/35 mm dish, the cultures fed

twice weekly and maintained for 1-2 weeks before toxin treatment. Neuronal and astrocyte cell cultures were similarly treated as mouse cortical explants with control, MAM or MAM+ BG supplemented media and the tissue processed for O^6-methylguanine DNA adducts.

Mouse Neuronal and Astrocyte Cell Cultures

Primary mouse granule or astrocyte cell cultures were prepared from the cerebella of 6-8-day old neonatal C57BL/6 (wild type) or $MGMT^+$ mice by dissociating the tissue in BSS with 0.1% trypsin as previously described (Kisby, G. E. and Acosta, D., 1987). Cell cultures were prepared by diluting the cells with high potassium (25 mM) containing plating media (DMEM with 10% FCS, 10% HS, 600 mg/ml glucose and 30 μg/ml insulin) and seeded at a density of 0.1-0.2 x 10^6 cells/well of a 24-well plate pre-treated for 1h with (*neurons*) or without (*astrocytes*) poly-D-lysine (100 μg/ml). Neuronal and astrocyte cell cultures were fed twice weekly and maintained for 7 days before treatment with 10-1000 μM methylazoxymethanol acetate (MAM) or 0.1-1.0 μM nitrogen mustard (HN2) for 24h.

DNA Damage

DNA was extracted and purified from cell and tissue cultures and examined for the level of O^6-methyldeoxyguanosine adducts as previously described by Kisby *et al.* (Eizirik, D. L. and Kisby, G. E., 1995;Esclaire, F. *et al.*, 1999). Briefly, media over cultures was aspirated, the tissue washed 3x with ice-cold PBS (pH 7.4) and homogenized in digestion buffer. Tissue samples were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) and DNA pelleted with 7.5 M NH_4Ac and cold absolute ethanol. The pellet was dissolved in H_2O, treated with DNase-free RNase for 1h, and the purified DNA pelleted with ice-cold absolute ethanol. Purified genomic DNA from MAM-treated mouse cortical explants and primary rat cortical astrocyte and cerebellar granule cell cultures was assayed by immunoslot blot for DNA adducts using a specific monoclonal antibody to O^6-methyldeoxyguanosine (EM-21; provided by Dr. G. Eberle, University of Essen). Calf thymus DNA and purified DNA from

control and MAM-treated cultures were diluted with H_2O to give a final concentration of 30 μg/ml. Alkylated DNA was diluted with control DNA or used directly (9.0 μg final concentration) and loaded onto a cellulose membrane (BA 85, S&S). Alkylated (1.0 mM *N*-methylnitrosourea) calf thymus DNA (0-2.7 μg) was diluted with untreated calf thymus DNA (9.0 μg final concentration) to develop a standard curve that was run along with samples. Membranes were baked at 80°C for 2h, blocked at room temperature and incubated overnight with EM-21 (0.2 μg/ml) at 4°C. Membranes were washed 4x with buffer (160 mM NaCl, 0.01% Triton X-100 in PBS) and incubated with blocking solution containing 25 μCi purified secondary antibody (^{125}I-IgG, Amersham) for 1h at room temperature. Membranes were washed 4x with buffer, blotted dry, wrapped in plastic wrap and exposed to X-ray film for 24-72 h. Specific binding of the primary antibody to the DNA adduct was visualized by scanning densitometry of autoradiographic films (Molecular Analyst™, BioRad) and the O^6-methylguanine levels in samples determined from a standard curve (r = 0.97) of alkylated DNA run along with the samples. Values are expressed as fmol O^6-methyldeoxyguanosine (O^6-mdG)/μg DNA.

Cell Counts

Mouse neuronal and astrocyte cell cultures treated with control media or media supplemented with various concentrations of MAM or HN2 were examined by light and fluorescence microscopy for cell viability using the fluorochromes propidium iodide (PI) and calcein-AM as previously described by Kisby *et al.* (Kisby, G. E. *et al.*, 2000) and Meira *et al.* (Meira, L. B. *et al.*, 2001). Briefly, the media over control, MAM or HN2-treated cultures was removed, replaced with control media containing 3 μM PI (a marker of cell damage) and 0.26 μM calcein-AM (a marker of cell viability), and the cultures treated for 10 min in a humidified 5%CO_2/O_2 incubator. The fluorochrome containing media was aspirated, the cultures washed once with control media, photomicrographs taken of the cell monolayer by epifluorescence microscopy, and 35 mm slides from each treatment group scanned and the numbers of calcein-AM and PI cells counted manually from each image. A second observer replicated all manual counts to

ensure count accuracy and minimal interobserver variability (< 1%).

LDH Activity

Neuronal cell injury was determined 24h after treatment with MAM by measuring LDH released from damaged cells by a standard kinetic assay for pyruvate. Briefly, media over control and treated cultures was removed, placed in a microcentrifuge tube, and stored at 4°C until analyzed for LDH activity. An aliquot (100 µl) of cell culture media from control or MAM treated neuronal cultures or plating media (blank) was incubated with 200 µl NADH (25 mg/ml), 200 µl sodium pyruvate (1 mg/ml) and 2.5 ml of 0.1 M phosphate buffer (pH 7.4) by measuring the decrease in absorbance of NADH at 340 nm over a 3-min time period at 30-s intervals as previously described by Kisby *et al.* (Kisby, G. E. and Acosta, D., 1987). LDH activity was reported as Units/ml for samples and the values corrected by subtracting the blank value.

Mitochondrial Function

Alamar blue™ (Trek Diagnostic Systems, Inc.) is a non-toxic metabolic indicator of viable cells that becomes fluorescent upon mitochondrial reduction and has been widely used to measure mitochondrial function in different cell systems (including neurons)(White, M. J. *et al.*, 1996;Springer, J. E. *et al.*, 1998). Mitochondrial function was determined in MAM treated neuronal cell cultures by adding Alamar Blue™ to a final concentration of 10% and the cells incubated at 37°C in a humidified 5%CO_2/O_2 incubator for 4h. Viability was measured when the medium in control wells turned blue to pink, typically at ~4h for granule cell neurons and astrocytes. Alamar blue™ fluorescence was measured in a FLUOstar™ (BMG LabTechnologies) automated plate-reading fluorometer, with excitation at 530 nm and emission at 590 nm. Values are reported as % redox activity of controls.

Western Blotting

Cell Cultures

Mouse cortical explants, rat cortical astrocytes and cerebellar granule cell cultures were examined by western blotting for the level of alkylguanine alkyltransferase (MGMT) or apurinic/apyrimidinic endonuclease (APE) protein using a monoclonal antibody against human MGMT (MT3.1, provided by Dr. T. Brent) or a polyclonal antibody to human APE (HAP1, provided by Dr. D. Henner, OHSU) according to previously published methods (Kisby, G. E. *et al.*, 1997;Esclaire, F. *et al.*, 1999). Briefly, extracts were prepared by homogenizing tissue or cells in lysis buffer, the lysed homogenate centrifuged, and the supernatant applied to a 10% SDS-PAGE-gel. The gel was electrophoresed and the proteins transferred onto a PVDF membrane. The blotted membranes were treated with blocking solution [5% BSA, 20 mM Tris-HCl (pH 8.2), and 0.9% NaCl] for 1 h at 37°C (*MGMT*) or at room temperature (*HAP1*). Membranes were immunoprobed by incubating blots with a solution containing 10 mM Tris HCl (pH 8.2), 0.1% BSA, 0.9% NaCl, 1% normal goat serum, and 10 μg/ml MT3.1 or HAP1 (1:1500 dilution) for 1-2 h at room temperature. Specific binding of MT3.1 or HAP1 to the repair proteins was visualized using either an Auroprobe™ secondary antibody kit (Amersham) with silver enhancement or a HRP conjugated goat anti-rabbit antibody and Enhanced Chemiluminescence (ECL™, Amersham) according to the manufacturers' instructions.

Mutant Mice

Wild type and $MGMT^+$ mice (6-8 days old) were killed by CO_2 inhalation, the brains dissected, and immediately snap frozen in liquid N_2. Frozen brain tissue was homogenized in lysis buffer [50 mM Tris (pH 7.5), 2 mM EDTA, 0.1 M NaCl, 1 mM dithiothreitol, and 200 μM phenylmethylsufonyl fluoride], sonicated for 90 s on ice, centrifuged at 3,000 x *g* for 10 min at 4°C, and the supernatant analyzed for protein concentration (Bradford). Protein extracts (25 μg) were applied to a 12% SDS-PAGE gel, the gel electrophoresed, the samples transferred onto nitrocellulose membranes, and the membrane immunoprobed with a monoclonal antibody to

MGMT (MT 22.1, a kind gift from Dr. T. Brent, St. Jude's Hospital, Memphis, TN). The mAb MT 22.1 was selected out of a panel of mAbs to MGMT (provided by Dr. D. Bigner, Duke University) because this antibody reacted strongly with both the mouse and human MGMT protein (GK, *unpublished data*). Bands were detected by incubating the membrane with an HRP-conjugated goat anti-mouse antibody, enhanced chemiluminescence (ECL™, Amersham), and phosphor image analysis of the blot as previously described by Kisby *et al.* (Kisby, G. E. *et al.*, 1997). Sets of biotinylated and kaleidoscope markers were also run along with the samples.

MGMT Activity

The activity of O^6-methylguanine-DNA methyltransferase (MGMT) in brain tissue extracts was measured as the removal of the [^{3}H]-methyl adduct from the O^6-position of guanine in [^{3}H]-methyl DNA alkylated with [^{3}H]*N*-methylnitrosourea ([^{3}H]MNU) as previously described (Lee, C.-K. *et al.*, 1999) **(Gerson, S. L. *et al.*, 1986).** An aliquot of wild-type (250 μg) or MGMT$^+$ (100 μg) brain tissue extract was incubated with [^{3}H]-methyl containing DNA in assay buffer for 60 min at 37°C (Gerson, S. L. *et al.*, 1986). The reaction was stopped by the addition of 50% TCA and samples incubated at 4°C for 30 min. The precipitate was collected by centrifugation, washed with 80% ethanol and the pellet hydrolyzed with 0.1 N HCl at 80°C for 60 min. The reaction was stopped by the addition of 0.01 M Tris, the hydrolyzed purines present in the supernatant separated by HPLC and the radioactivity quantified using a liquid scintillation counter.

Statistical Analysis

Data are expressed as the mean ± S.E.M. All data obtained were evaluated for statistical significance by one-way analysis of variance (ANOVA). A probability value of $p < 0.05$ was considered significant unless otherwise noted.

RESULTS

MGMT Inhibitor Studies

MAM damages DNA to produce multiple DNA adducts (i.e., O^6-methylguanine, N^7-methylguanine, 8-methylguanine), but the specific DNA adduct that may be responsible for MAM-induced neurotoxicity is not known. Since N^7-methylguanine DNA adducts do not show marked miscoding or cytotoxic properties (Lindahl, T., 1993), initial studies focused on the role of O^6-methylguanine DNA adducts in MAM-induced neurotoxicity. For these studies, mouse cortical explants were pre-treated with 5.0 μM O^6-benzylguanine (BG), a potent inhibitor of O^6-methylguanine methyltransferase (MGMT) (Dolan, M. E. *et al.*, 1985;Dolan, M. E. *et al.*, 1990), before treatment with 100 μM MAM and the cultures examined for O^6-methylguanine and MGMT levels (**Figure 1**) or cell viability (**Figure 2**), respectively. In non-nervous tissue (human colon tumor cells), this concentration of BG is reported to inhibit MGMT without causing overt toxicity (Seeley, M. R. and Faustman, E. M., 1998) while higher concentrations of the inhibitor (> 500 μM) are cytotoxic to cultured rat cerebellar neurons (Mehl, A. *et al.*, 2000). Immunoslot blot is a very sensitive (*e.g.* 1 modified DNA base in 10^7-10^8 parent bases) (Nehls, P. *et al.*, 1984) and specific technique for the identification of damaged DNA and was used to determine the level of O^6-methylguanine DNA adducts (**Figure 1A**). As in previous studies with rat cortical neurons (LeDoux, S. P. *et al.*, 1996;Kisby, G. E. *et al.*, 1999), O^6-methylguanine was detected in genomic DNA purified from cerebellar neurons treated with 100 μM MAM (372 fmol/μg DNA), but the levels in comparably treated astrocytes were below the limit of detection (<70 fmol/μg DNA). A significant amount of O^6-mdG (~ 81 fmol/μg DNA) was also detected in MAM treated explant cultures, but the levels were ~ 2x higher (~156 fmol/μg DNA) in explants pre-treated with BG (**Figure 1A**). Consistent with these findings, MGMT levels were ~ 12-fold or 4-fold lower in murine cortical explants and rat astrocyte cell cultures pre-treated with the inhibitor (**Figure 1B**). However, O^6-benzylguanine (BG) was not selective for MGMT because

levels of the base-excision DNA repair protein apurinic/apyrimidinic endonuclease (APE) were also altered by the inhibitor with levels ~2x lower or higher in comparably treated explants or astrocytes, respectively (**Figure 1B**). The influence of BG on neural tissue APE levels may explain the increased sensitivity of BG treated differentiated P19 cells to methanesulfonate (Seeley, M. R. and Faustman, E. M., 1998), an alkylating agent that produces predominantly N^7-alkylguanine DNA adducts. The difference in O^6-methylguanine levels between these two CNS cell types is likely due to higher basal levels of MGMT in astrocytes (LeDoux, S. P. *et al.*, 1996;Kisby, G. E. *et al.*, 1999).

Explants treated with MAM and O^6-benzylguanine were also examined for cell viability using the vital fluorochromes calcein AM and propidium iodide (**Figure 2**). All explants appeared viable (uniform green fluorescence) before toxin treatment (0-day) (**Figure 2A-C**) or when treated in a similar manner with 5.0 μM guanine•HCl (*data not shown*). As expected, tissue injury was more extensive in explants treated with both O^6-benzylguanine and MAM (**Figure 2F**) than in explants treated with control (**Figure 2D**) or MAM (**Figure 2E**) supplemented media. Similar results have been obtained for differentiated murine P19 cells that were pre-treated for 24h with 2.5-10 μM BG before exposure to the related alkyating agent *N*-methylnitrosourea (Seeley, M. R. and Faustman, E. M., 1998). Unexpectedly, O^6-benzylguanine was moderately toxic to explant cultures and tissue injury was comparable to explants treated with MAM (*data not shown*) suggesting that MGMT may have other functions in mature nervous tissue. Therefore, O^6-benzylguanine potentiated MAM-induced neurotoxicity by lowering MGMT in post-mitotic neurons and increasing the cytotoxic levels of O^6-mdG DNA adducts. However, O^6-benzylguanine also perturbed nervous tissue APE suggesting that the neurotoxic effects of MAM could have resulted from one or more DNA adducts (i.e., O^6-mdG and/or N^7-mdG) or from the indirect action of O^6-benzylguanine on neural function. Therefore, neuronal cells from mice that are selectively proficient (or deficient) in MGMT could help define

the role of O^6-mdG DNA adducts in MAM-induced neurotoxicity. For these studies, cerebellar neuronal and astrocyte cell cultures were prepared from mice that overexpress MGMT and the cultures examined for cell viability. Comparable studies are now underway using MGMT knockout mice and will be reported separately.

MGMT Levels and Activity in Mutant Mice

Initial studies compared the relative distribution and activity of O^6-methylguanine methyltransferase (MGMT) among different brain regions of wild type mice and mice that overexpress human MGMT ($MGMT^+$). hMGMT (~22-23 kDA) was detected (*black arrow*) by western blotting in the cortex, hippocampus, midbrain, cerebellum and brainstem of $MGMT^+$ mice, but not in brain tissue of wild-type mice (**Figure 3**, *top*). As expected, the endogenous mouse protein (*white arrow*) was detected in both wild type and $MGMT^+$ mice. Consistent with these observations, MGMT activity was higher in the cerebellum (~50-fold) and other brain regions (12 to 80-fold) of $MGMT^+$ mice when compared with similar brain regions of wild-type mice (**Figure 2**, *bottom*). Therefore, the level and activity of MGMT is elevated in cerebellar neurons and astrocytes of $MGMT^+$ mice. Interestingly, MGMT activity was higher in the brain of $MGMT^+$ mice when compared with the activity previously reported for other organs (e.g., liver, kidney) suggesting that the protein may have other functions in the CNS.

Viability of MAM Treated Murine Neurons

The toxic properties of methylazoxymethanol (MAM) were examined in cerebellar neuronal and astrocyte cell cultures prepared from mice that overexpress human O^6-methylguanine-DNA alkyltransferase ($MGMT^+$). The sulfur mustard analogue mechlorethamine (HN2, nitrogen mustard) was used for comparison because this genotoxin produces N^7-alkyl DNA adducts and cross links, but not O^6-methylguanine-DNA adducts (Osborne, M. R. *et al.*, 1995;Lawley, P. D. and Phillips, D. H., 1996). The toxicity of MAM and the related genotoxin HN2 was examined in cerebellar neurons and astrocytes using the vital fluorochromes calcein-AM (*green*

fluorescence) and propidium iodide (PI, *red fluorescence*) (**Figures 4**). Calcein-AM is a cell permeant dye that is taken up by active mitochondria while a loss in membrane integrity leads to uptake of PI and nuclear staining of dying cells. Moderate cell loss was observed in wild-type mouse cerebellar neurons treated with 100 μM MAM (40-50%) and 1.0 μM HN2 (40%). In contrast, $MGMT^+$ cerebellar neurons were protected from 100 μM MAM, but not from 1.0 μM HN2-induced cytotoxicity. $MGMT^+$ cerebellar neurons were also protected at higher concentrations of MAM as indicated by the significantly lower LDH activity measured in cell culture media (**Figure 5**, *right*) or the higher mitochondrial redox activity (**Figure 5**, *bottom*) than comparably treated wild-type cells. In comparison to mouse cerebellar neurons, wild type or $MGMT^+$ astrocytes were relatively insensitive to 100 μM MAM or 1.0 μM HN2-induced cytotoxicity (**Figure 4**). In fact, concentrations greater than 10 μM HN2 or 500 μM MAM were required to induce significant cell loss (>50%) in astrocyte cultures (*data not shown*). Similar results have also been reported for studies that compared the relative toxicity of HN2 in rat cerebellar neuronal and astrocyte cell cultures (Kisby, G. E. *et al.*, 2000), an effect probably related to the high basal levels of DNA repair in these neural cells (LeDoux, S. P. *et al.*, 1996).

DISCUSSION

The genotoxin MAM has been widely used by neurobiologists to selectively ablate neurons in the developing nervous system of rodents (Balduini, W. *et al.*, 1986;Cattabeni, F. and Di Luca, M., 1997). In addition to the well-studied anti-mitotic effect of MAM on the developing nervous system, MAM induces long-lasting abnormalities in post-mitotic neurons. These include persistent inhibition of neurite outgrowth after treatment of hippocampal neurons for 0-1 days with low micromolar concentrations (0.1 nM – 1.0 μM) of MAM (Hoffman, J. R. *et al.*, 1996) or increased *tau* mRNA expression and delayed cell death after brief treatment of rat cortical neurons with 10 μM MAM (Esclaire, F. *et al.*, 1999). Hippocampal neurons treated with MAM failed to grow even after media over the cells was replaced with glial conditioned media suggesting that the effects of the genotoxin on post-mitotic neurons are persistent. In more recent studies, MAM was cytotoxic to post-mitotic cortical neurons and the neurons that survived had elevated*tau* gene expression, DNA damage (i.e., N^7-methylguanine, O^6-methylguanine), and reduced DNA repair levels and activity. Although MAM is proposed to only affect actively dividing neurons and spare both premitotic or migrating postmitotic neurons (Cattabeni, F. and Di Luca, M., 1997), these recent findings demonstrate that the genotoxin also targets post-mitotic neurons, possibly by a similar DNA damage mechanism.

MAM like other DNA alkylating agents reacts with DNA to form a number of DNA adducts, with O^6-mdG being the major mutagenic and cytotoxic DNA adduct. In contrast, N^7-methylguanine DNA adducts are the primary DNA lesion that is formed by methylating agents, but this type of DNA damage is considered non-lethal or mutagenic (Lindahl, T., 1993). The purpose of the present studies was to further explore the '*slow toxin*' or DNA damage hypothesis of MAM by examining the cytotoxicity of MAM in nervous tissue with perturbed DNA repair. Two different approaches were taken to manipulate nervous tissue DNA repair, treatment of nervous tissue with the well-established MGMT inhibitor O^6-benzylguanine (BG) (Dolan, M. E.

et al., 1985;Dolan, M. E. *et al.*, 1990) and overexpression of human MGMT in murine brain by gene targeting (Gerson, S. L. *et al.*, 1994). In rodents, nonhuman primates and humans, O^6-benzylguanine (BG) is metabolized to the more stable and potent AGT inhibitor 8-oxoBG by human P450-mediated or cytosolic aldehyde oxidases (Ewesuedo, R. B. *et al.*, 2001). A concentration of 2.5 – 10 μM BG has been shown to significantly reduce MGMT levels in murine P19 embryonic cells differentiated with retinoic acid (cells that are biochemically and morphologically very similar to mature CNS neurons) and to increase their sensitivity to various methylating agents (Seeley, M. R. and Faustman, E. M., 1998). However, a combination of BG and various alkylating agents failed to induce cytotoxicity or inhibit differentiation in proliferating neurons grown in micromass cultures prepared from the striatum of rat embryos (Kidney, J. K. and Faustman, E. M., 1995). Yet, the effect of BG is reportedly more persistent on brain AGT than any other organ (e.g., kidney, liver) examined (Ewesuedo, R. B. *et al.*, 2001). These findings suggest that the proliferative state of a cell plays an important role in determining the susceptibility of nervous tissue to alkylating agents. Consistent with these findings, the present studies examined explants cultured from the mouse motor cortex or rat cerebellar neurons and cortical astroctyes to examine the relationship between DNA damage and MAM-induced toxicity among different CNS cell types. As previously reported in differentiated murine P19 cells (Seeley, M. R. and Faustman, E. M., 1998), the MGMT inhibitor BG reduced cellular MGMT levels and this reduction increased the sensitivity of murine explants to MAM-induced DNA damage and neurotoxicity. These findings suggest that MAM-induced O^6-methylguanine DNA adducts play an important role in MAM-induced neurotoxicity. However, the concomitant reduction of apurinic/apyrimidinic endonuclease (APE) by BG, a protein involved in the repair of MAM-induced N^7-methylguanine DNA adducts, could have contributed to the increased susceptibility of nervous tissue to the genotoxin. The equitoxic effect of MMS (produces predominantly N^7-methylguanine DNA adducts) and MNU to differentiated P19 cells is evidence in support of this hypothesis. Consequently, studies were conducted using neuronal cultures from mice that overexpress MGMT to determine if O^6-methylguanine DNA adducts

play an important role in MAM-induced neurotoxicity. We demonstrate that overall cell survival and mitochondrial function were essentially preserved in MAM treated murine neurons that overexpress MGMT, results strongly supporting a role for O^6-mG DNA adducts in MAM-induced neurotoxicity. This hypothesis is further supported by preliminary studies demonstrating that cerebellar neurons from MGMT deficient mice are more sensitive to MAM-induced cytotoxicity than comparably treated *Aag*- deficient mice (GK, *unpublished data*). Moreover, cell survival was comparable in wild type or $MGMT^+$ overexpressing neurons treated with HN2 because this related alkylating agent does not produce O^6-methylguanine DNA adducts. Conversely, cerebellar neurons from nucleotide excision repair (NER) deficient mice are more sensitive to HN2 and UV-irradiation, but equitoxic as wild type cells to MAM (GK, *unpublished data*). These findings suggest that O^6-methylguanine DNA adducts play an important role in MAM-induced neurotoxicity. Additional studies with cerebellar neurons from mice with different DNA repair deficiencies (e.g., MGMT, Aag) are currently underway to confirm these findings.

In summary, findings from the present work suggest that perturbing DNA repair (i.e., MGMT) *via* pharmacological agents or gene targeting can influence the sensitivity of post-mitotic nervous tissue to environmental genotoxin agents. More importantly, these preliminary studies suggest for the first time that specific DNA adducts are involved in MAM-induced neurotoxicity. Additional studies are underway to confirm these findings and to explore the potential role of other DNA repair pathways (i.e., base-excision, nucleotide excision) in protecting neurons from MAM-induced toxicity. Results from these studies will provide a better understanding of the mechanism by which MAM targets neurons and its putative role in neurodegenerative disease.

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Figure Legends

Figure 1. Effect of O^6-benzylguanine (BG) on DNA damage and DNA repair in murine cortical explants or rat neuronal and astrocyte cell cultures treated with MAM. A. Aliquots of alkylated DNA standards (0-6 pmol O^6-methyldeoxyguanosine/μg DNA) and purified DNA from mouse cortical explants treated with 100 μM MAM and BG or rat neuronal and astrocyte cell cultures treated with 100 μM MAM were examined by immunoslot blot for O^6-methyldeoxyguanosine (O^6-mdG) levels. B. Western blots of O^6-methylguanine methyltransferase (MGMT) or apurinic/apyrimidinic endonuclease (APE) levels in protein extracts from control or O^6-benzylguanine (BG) treated mouse cortical explants (20 μg), rat cortical astrocytes (15 μg) or cerebellar neurons (20 μg). MGMT and APE levels were determined using a monoclonal antibody to human MGMT (MT3.1) or a polyclonal antibody to human APE (HAP1), respectively. MGMT was visualized using an Auroprobe™ secondary antibody kit (Amersham) with silver enhancement according to the manufacturer's protocols. HAP1 was visualized using an alkaline-phosphatase conjugated anti-rabbit secondary antibody and colorimetric detection according to the manufacturer's protocols (Protoblot™ II AP system, Promega). M=markers, hMGMT= recombinant human MGMT (1.0 ng).

Figure 2. Effect of the MGMT inhibitor O^6-benzylguanine (BG) on MAM-induced cytotoxicity in murine cerebral cortical explants. Fluorescent micrographs from mature mouse cortical

explants before (**A,B,C**) and after continuous treatment for 5-days with control media (**D**) or media supplemented with 100 μM MAM (**E**), or 100 μM MAM + 5.0 μM BG (**F**). Treated explants were pre-incubated for 30 min with the fluorochromes rhodamine 123 (*green fluorescence*; viable cells) and propidium iodide (*orange-red fluorescence*; dead cells) and examined by fluorescence microscopy. Mags: X9.

Figure 3. O^6-Methylguanine methyltransferase (MGMT) levels and activity in different brain regions of wild type and MGMT overexpressing ($MGMT^+$) mice. Protein extracts (25 μg) from postnatal (6-8 day old) brain tissue of wild and $MGMT^+$ mice were electrophoresed on a SDS-PAGE gel and the membrane blocked and immunoprobed with a monoclonal antibody to MGMT (MT 22.1) [A]. Specific binding of MGMT was visualized using an HRP-conjugated goat anti-mouse antibody (1:1000) and enhanced chemiluminescence. For MGMT activity, an aliquot of protein extract (100 μg or 250 μg) was incubated with [^{3}H]methyl DNA and the amount of [^{3}H] O^6-methylguanine determined by HPLC with liquid scintillation counting [B]. Values are the mean ± SEM. Std= biotinylated markers. Human MGMT (*black arrow*) or mouse MGMT (*white arrow*). CX=cortex; HP=hippocampus; TH=thalamus; MB= midbrain; CB= cerebellum; BS=brainstem.

Figure 4. Photomicrographs of representative fields from wild type and $MGMT^+$ neuronal and astrocyte cell cultures treated with MAM or nitrogen mustard (HN2). Cerebellar granule and astrocyte cell cultures from wild type and $MGMT^+$ littermates were treated with 100 μM MAM or 1.0 μM HN2 for 24h, the culture media removed and the cells incubated for 30 min with culture media containing the vital fluorochromes calcein-AM and propidium iodide.

Figure 5. Viability of MAM and HN2 treated cerebellar neurons from wild type and $MGMT^+$ mice. Mouse cerebellar granule cell cultures were treated with various concentrations of HN2 (0.1 μM, 1.0 μM) or MAM (10 μM - 1000 μM) for 24h, an aliquot of the culture media analyzed

for LDH activity, the cultures incubated for 4h with Alamar Blue™ and examined for fluorescence. After 4h, the cultures were incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide). Cell viability was assessed by counting the total number of live (*green*) and dead (*red*) cells of fluorescent photomicrographs taken from 3 random fields (~500-1000 cells/field) of each well, as previously described by Kisby *et al.* (Kisby, G. E. *et al.*, 2000) and Meira *et al.* (Meira, L. B. *et al.*, 2001). Values represent the mean ± SEM ($n= 4$). Significantly different from MAM treated wild-type cells ($* p < 0.05$, $** p < 0.01$, $*** p < 0.001$, ANOVA).

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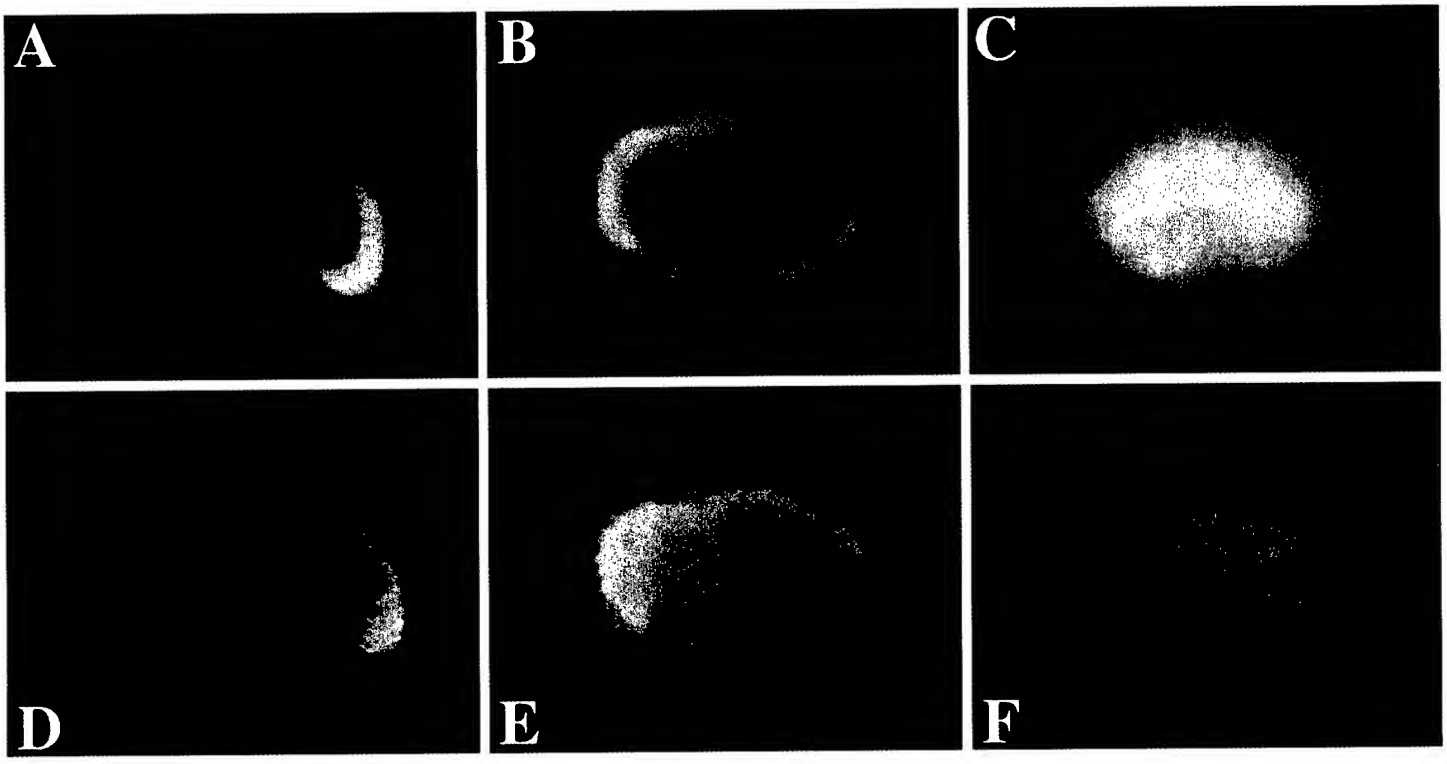
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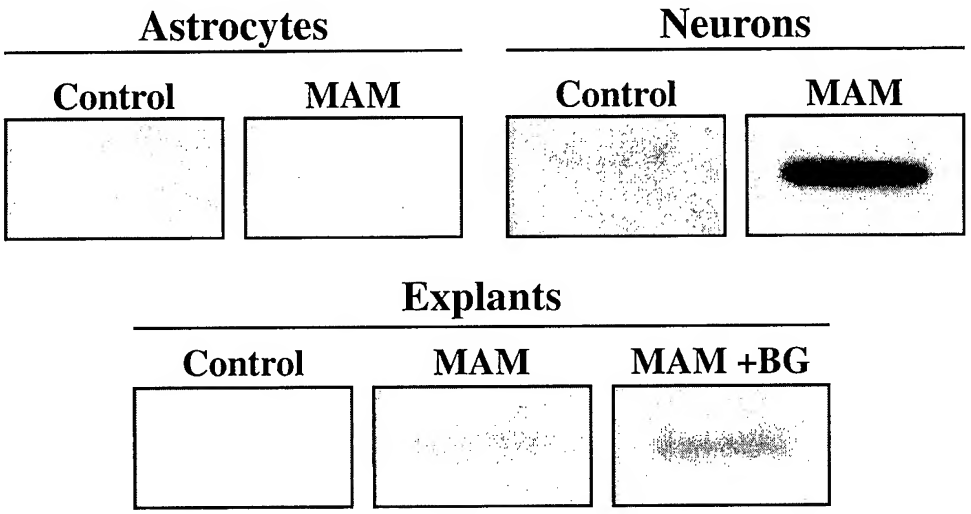
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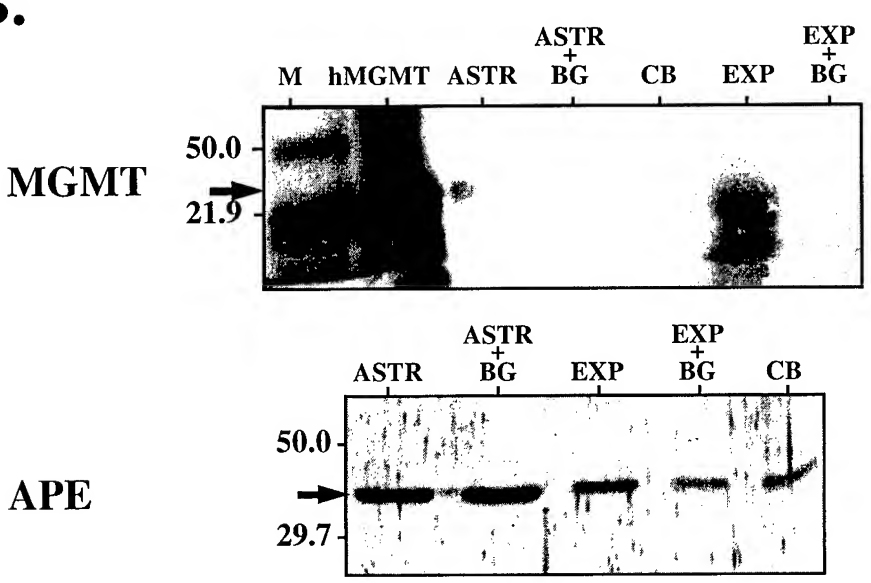
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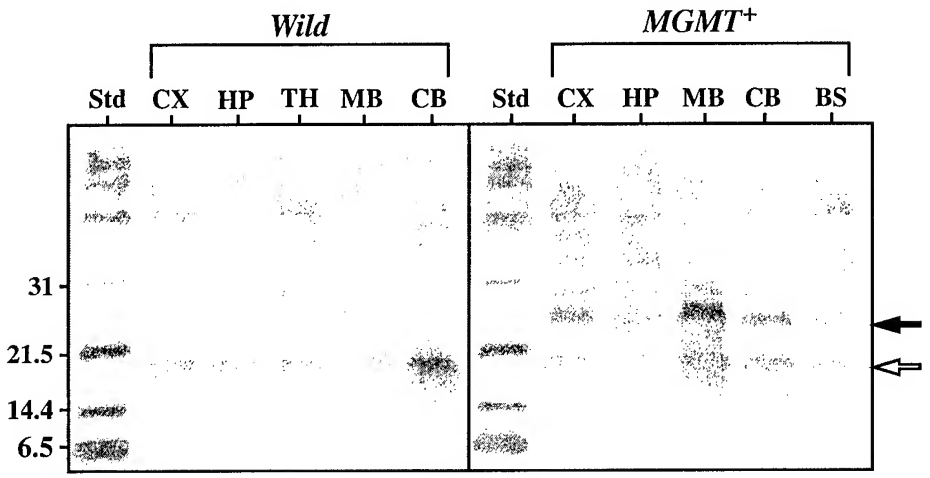
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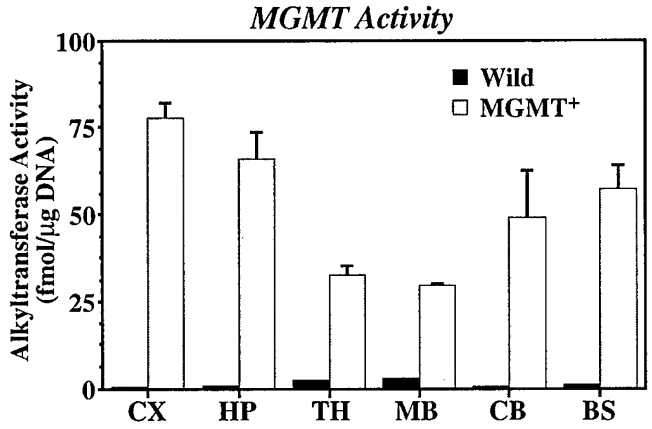
B.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Alkyltransferase Activity
(fmol/μg DNA)
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75
50
25
0
Wild
MGMT+
CX
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TH
MB
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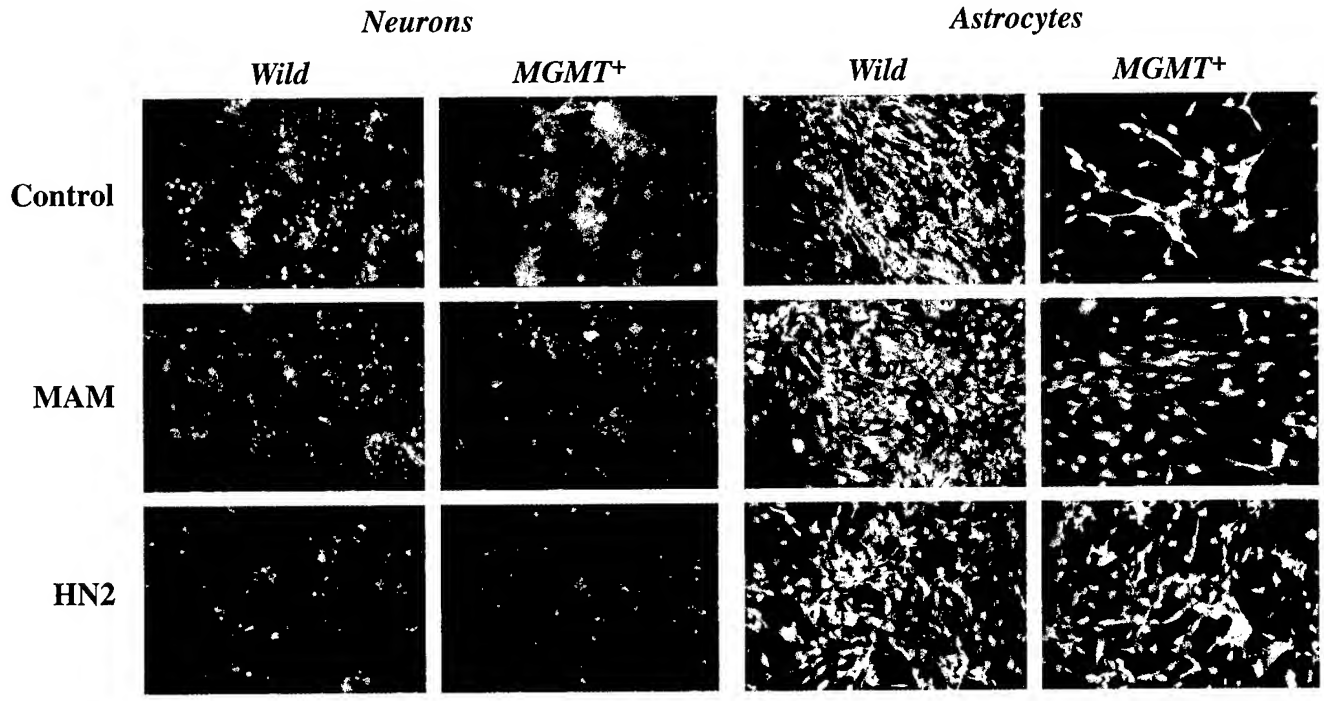
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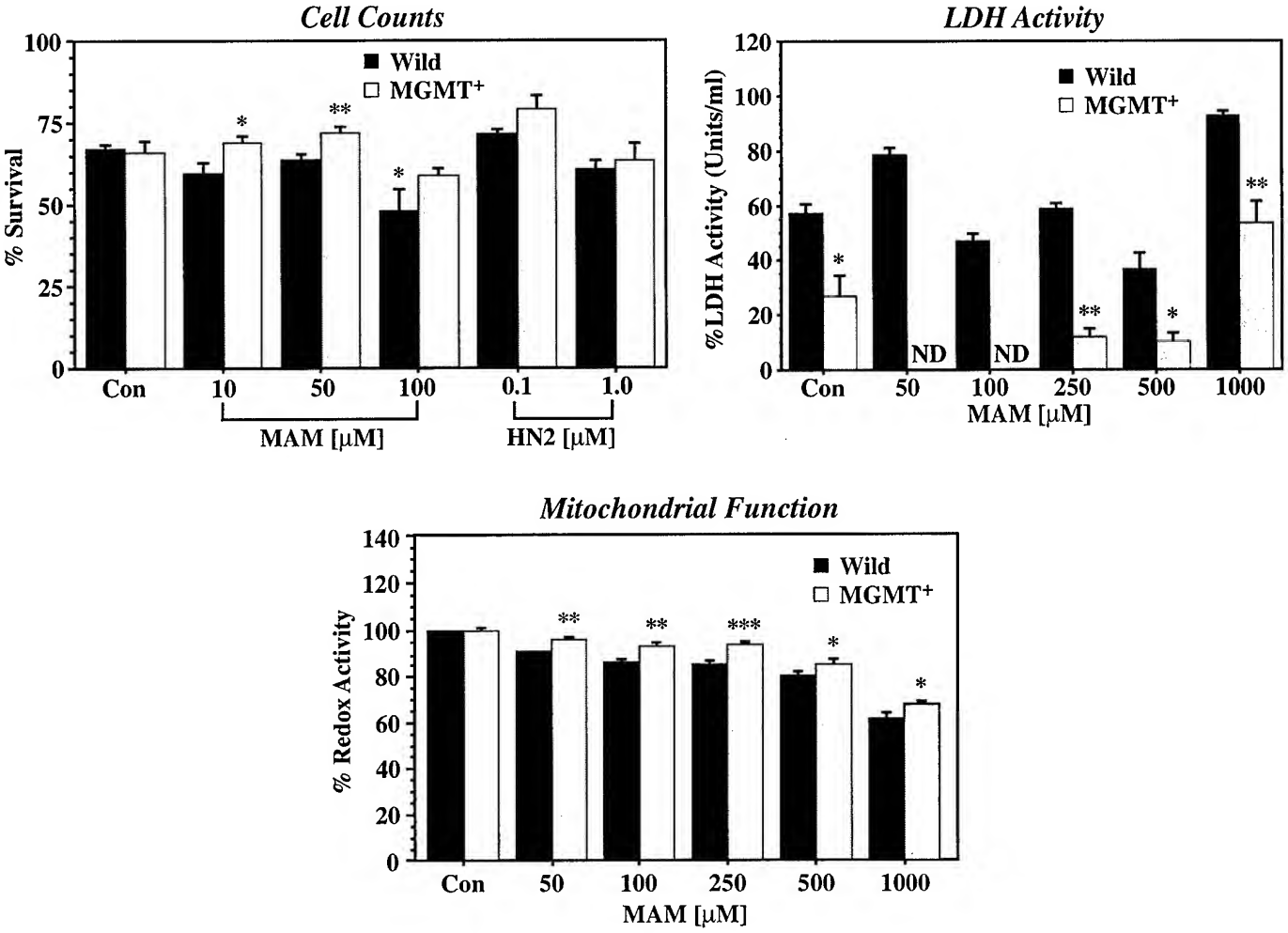
Astrocytes
Wild
MGMT+
Wild
MGMT+
Control
MAM
HN2


Cell Counts
% Survival
Wild
MGMT+
Con
10
50
100
0.1
1.0
MAM [μM]
HN2 [μM]
LDH Activity
%LDH Activity (Units/ml)
ND
ND
Con
50
100
250
500
1000
MAM [μM]
Mitochondrial Function
% Redox Activity
Con
50
100
250
500
1000
MAM [μM]

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DRAFT VERSION

DNA repair in the nervous system

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Introduction

It has been proposed that the etiology of chronic neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD) involves an accumulation of DNA damage because of an underlying defect in DNA repair (Bradley and Krasin, 1982; Robbins 1983, Mazzarello et al. 1992, Rolig and McKinnon 2000). This hypothesis receives strong support from the observation that characteristic features of chronic neurodegenerative disease, that is the progressive loss of neurons and associated neuropathological changes, also occur in the brains of patients with the rare hereditary DNA repair disorders ataxia telengiectasia (AT) and xeroderma pigmentosum (XP) (Gatti et al. 1991, Itoh et al. 1999, Larnaoutet al. 1998, Tandan et al. 1987). A better understanding of the molecular mechanisms of DNA repair in the nervous system will be required to determine the significance of DNA damage and its repair in rare hereditary DNA repair disorders and chronic neurodegenerative disease.

The importance of DNA repair for neuron maintenance is supported from three lines of evidence: 1) the observation that the efficiency of DNA repair can be modulated in neurons, 2) the identification of DNA repair alterations in neurodegenerative diseases, and 3) neurological symptoms are often a characteristic feature of diseases caused by deficiencies in DNA repair. This review examines these three topics.

DNA repair pathways

Damage to genomic DNA can result from numerous chemical, physical and endogeneous sources. In the first category are alkylating agents and oxidative compounds that cause minor modification of the structure of DNA bases. Other kinds of chemicals covalently bind to DNA and perturb DNA-processing enzymes, like polymerases, by steric hindrance or by forming inter- or intra-strand cross-links. Physical agents include ultraviolet (UV) light that generate pyrimidine dimmers repaired by the same pathway as bulky adducts (which makes them a convenient model lesion even though the central nervous system (CNS) is not likely to be subject to UV irradiation) and ionizing radiation that can cause both strand breaks and oxidative damage. Finally, important endogeneous sources of DNA damage are the reactive oxygen species (ROS) produced by the mitochondrial oxidative phosphorylation system. This is likely a major problem for neurons, due to their high oxygen metabolism.

To deal with DNA alterations, human cells elicit a battery of DNA repair systems: direct repair (DR), nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), strand-break repair by homologous recombination (HR) and by non-homologous end-joining (NHEJ), and post-replicative recombination repair (PRR), to name the principal categories. This review will concentrate on BER and NER and on the role of a special pathway that deals with lesions in expressed genes, transcription coupled repair (TCR).

BER deals mainly with small modifications of the DNA bases, such as oxidations or methylations. In general, a given lesion is recognized by a glycosylase, a fairly specific enzyme that cleaves the damaged or inappropriate base from the deoxyribose sugar moiety, leaving the deoxyribose-phosphate backbone intact. Such apurinic/apyrimidinic sites are known as AP sites. A second enzyme, called AP-endonuclease, recognizes AP sites and cuts the backbone, allowing the base-less sugar to be removed. The resulting gap can then be filled by a DNA polymerase and sealed by a ligase (Lindahl and Wood, 1999).

NER is a more versatile system that can recognize a vast array of lesions, from UV-induced pyrimidine dimers to bulky chemical adducts and inter-strand cross-links. The lesion recognition is probably based on distortions of the DNA double helix and is performed by a small set of recognition enzymes: XPC, XPE, XPA, and RPA. A denaturation bubble of about 29 nucleotides is opened around the lesion through the action of the helicases XPB and XPD, which are subunits of the RNA polymerase II (RNAP II) basal transcription factor TFIIH. The strand bearing the damage is then incised at each end of the bubble, by the structure-specific enzymes XPG on the 3' side of the lesion, and the heterodimer XPF/ERCC1 on the 5' side. A short oligonucleotide containing the lesion is

removed and the resulting gap is filled by a DNA polymerase and sealed by a ligase (Lindahl and Wood, 1999).

In part because cells need them more urgently and in part because of the impasse of an arrested RNA polymerase at a lesion, transcribed genes are usually repaired more efficiently than the bulk of the genome (Hanawalt, 1994). This so-called transcription-coupled repair (TCR) pathway is confined to the transcribed strand of active genes and requires a number of specialized proteins, including CSA and CSB. The molecular details of TCR are unknown, but it is generally accepted that the damage sensor is RNAP II, that triggers the repair reaction when stalled at a lesion. TCR was originally described only for lesions repaired by NER; however it has recently been shown that several oxidative lesions normally repaired by BER, thymine glycol (Leadon and Cooper, 1993) and 8-oxoguanine (Le Page et al., 2000), are also subject to TCR.

DNA repair in neurons

BER in the CNS

The nervous system is a heterogeneous organ composed of distinct regions and specialized cells. A cardinal feature of the aging and/or diseased brain is the accumulation of DNA damage in specific regions and cell types, suggesting that there are regional and cellular differences in DNA repair. This hypothesis is supported by recent work from several groups indicating that the distribution of some BER enzymes is indeed heterogeneous within the rodent, primate and human nervous system (Duguid et al. 1995, Kisby et al. 1995b, Ono et al. 1995, Rivkees and Kelley 1994). Two DNA repair enzymes, O^6-methylguanine methyltransferase (MGMT) and AP-endonuclease (APE), are expressed at high levels in the cerebellum and at low levels in forebrain structures (Duguid et al. 1995, Wiestler et al. 1984, Wilson et al. 1996, Woodhead et al. 1985), and both are reported to decrease with age (Edwards et al. 1998, Mandavilli and Rao 1996b, Ono et al. 1995, Silber et al. 1996, Silber et al. 1993). Basal expression of these two proteins also differs among CNS cell types, with levels reportedly lower in neurons than in glial cells (Duguid et al. 1995, Kisby et al. 1995a, Kisby et al. 1995b, LeDoux et al. 1996, Korr and Schultz 1989). This heterogeneity should *a priori* make certain brain regions (e.g., cerebrum) and cell types (e.g., neurons) more vulnerable to insult by alkylating and oxidative DNA damaging agents.

Indeed, there is a differential sensitivity of the CNS to the nitrosourea class of alkylating agents. Viability studies demonstrated an increased sensitivity in both oligodendrocytes and microglia compared to astrocytes. The formation and repair of *N*-methylpurines within the mitochondrial DNA (mtDNA) of astrocytes, oligodendrocytes and microglia was assessed by a quantitative Southern blot procedure (LeDoux et al., 1998). This study revealed no differences in the initial formation of *N*-methylpurines within mtDNA among the three cell types. In contrast a significant decrease in repair capacity was observed in oligodendrocytes and microglia. DNA fragmentation and quantitative morphological analysis of ultrastructural changes indicated that the induction of apoptosis correlated with this decrease in repair capacity.

In a second study (LeDoux, et al., 1996), repair ofO^6-methylguanine in total cellular DNA from the three glial cell types was assessed using an immunoslot blot procedure and a monoclonal antibody that is specific for O^6-methylguanine. As with the *N*-methylpurines, repair was significantly greater in astrocytes compared to either oligodendrocytes or microglia. Consistent with this result, MGMT levels were elevated in astrocytes as compared to oligodendrocytes and microglia. Thus, it appears that repair of both O^6-methylguanine and *N*-methylpurines in mtDNA is more efficient in astrocytes than in the other two cell types. Additionally, the reduced repair capacity in oligodendrocytes and microglia again correlated with the induction of apoptosis.
Neurons are particularly vulnerable to DNA damage by reactive oxygen species (ROS) because of their high oxygen metabolism and susceptibility to certain pathological conditions. The oxidative stress that results has been implicated as a causal factor in a wide variety of neurodegenerative diseases. There are cell-specific differences in sensitivity to oxidative stress; oligodendrocytes and

neurons are extremely sensitive (Jurlick, 1997) and mitochondria appear to be the major intracellular source of ROS. It is estimated that 2% of the electrons that pass down the electron transport chain "leak off" and form superoxide. Menadione, which redox cycles with complex I of the mitochondrial electron transport chain to generate superoxide, is a widely used experimental agent to enhance cellular ROS levels. Mitochondrial DNA is particularly sensitive to menadione-induced DNA damage because of its close proximity to the inner mitochondrial membrane where ROS are generated and its lack of protection by histones. (cf. Ljungman and Hanawalt, 1992). Repair of oxidative DNA damage has been assessed in menadione-treated primary cultures of rat astrocytes, oligodendrocytes, microglia and cerebellar granule cells (Hollensworth et al., 2000). The repair capacity in astrocytes was very efficient, with all of the detectable damage removed by 6 hrs. Less efficient repair was observed with oligodendrocytes, microglia or cerebellar granule cells. In the same study, cell viability was assessed by trypan blue exclusion 24 hours after exposure to menadione. A correlation was demonstrated between decreased mtDNA repair and increased cell death.

Two pathways of cell death, necrosis and apoptosis, have been described. Apoptosis, as indicated by characteristic DNA fragmentation, quantitative EM morphological analysis, and phophatidylserine 'flipping' was observed in oligodendrocytes, microglia and neurons but not in astrocytes following exposure to agents that induce either alkylation or oxidative DNA damage. If one wants to be able to regulate this process, it is important to know which pathway is being activated. Although there are many different stimuli of apoptosis one pathway involves receptors on the plasma membrane and another involves the release of factors from the mitochondria. The mitochondrial pathway has been shown to include the release of cytochrome c from the intermitochondrial space into the cytoplasm and the activation of caspase 9. In the present case (Hollensworth et al., 2000), it appears that it is this pathway that was activated because cytochrome c release was observed to occur by western blot analysis. Subsequently, the same group demonstrated the activation of caspase 9 but not caspase 8 in oligodendrocytes, microglia and neurons. **Reference?** Thus, a correlation between decreased mtDNA repair capacity and the induction of apoptosis is demonstrated .

BER in the Aging Brain

The DNA damage hypothesis of aging predicts that DNA damage is elevated (Alam et al. 1997, Lyras et al. 1997, Mecocci et al. 1997, Rehman et al. 1999, Su et al. 1995) and DNA repair is reduced (Beal 2000, Edwards et al. 1998) in diseases of old age such as cancer, diabetes, and neurodegenerative disease (Mecocci et al. 1994, Mecocci et al. 1993). Since age is the most reliable and robust risk factor for neurodegenerative disease, the aging process may be an important factor that influences nervous tissue DNA repair and contributes to the accumulation of DNA damage. Consistent with this hypothesis, the oxidative DNA lesions 8-oxoguanine (8oxoG, also known as 8-hydroxydeoxyguanosine), thymine glycol, and apurinic sites accumulate with age in rodent (Bhaskar and Rao 1994, Fraga et al. 1990, Hirano et al. 1996, Mandavilli and Rao 1996a, Mandavilli and Rao 1996b, Mullaart et al. 1990, Mullaart et al. 1988, Zahn et al. 1996) and human tissues (Mecocci et al. 1993, Mecocci et al. 1997). These same DNA lesions are also reportedly elevated in the brains of individuals with ALS (Ferrante et al. 1997, Fitzmaurice et al. 1996), PD (Alam et al. 1997, Sanchez-Ramos et al. 1994) and AD (Gabbita et al. 1998, Lyras et al. 1997, Mecocci et al. 1994). Since these DNA lesions are predominantly repaired by the BER pathway, it is possible that this DNA repair pathway may be perturbed in the aging or diseased brain. Indeed, the expression and activity of the BER proteins APE and OGG1 (an 8-oxoG DNA glycosylase) were recently reported to be perturbed in the brains of aging rodents (Edwards et al. 1998) and primates (Kohama et al. 2000). Moreover, mice with a point mutation in the OGG1 gene exhibited a shortened life span, learning and memory deficits and biochemical changes (e.g., mitochondrial dysfunction, accumulation of single-strand breaks) consistent with features of oxidative stress in CNS and peripheral organs (Nishikawa et al. 1998, Choi et al. 1999, Hosokawa et al. 2000). These studies indicate that a defect in BER can produce features of accelerated aging within the CNS.

In primates, repair of oxidative DNA damage in the *substantia nigra* decreases with age, specifically in dopaminergic neurons that contain AP-endonuclease (APE), but not in neurons that contain the glycosylase OGG1 (Kohama et al. 2000). APE activity was also shown to be reduced with age in only certain regions of the primate brain (Kohama et al. 2000). A potential functional consequence of

this reduction or imbalance in DNA repair may be to trigger a cascade of events that lead to decreased neurotransmitter levels in major sites of innervation and cell injury. An imbalance in any one component of the BER pathway results in increased cell death (Coquerelle et al. 1995, Glassner et al. 1998), which may explain the cell loss observed in the *substantia nigra* of the aging brain. The seminal findings from these animal studies are that the aging process influences nervous tissue DNA repair and that it may be an important factor that contributes to the pathogenesis of neurodegenerative disease.

NER in neurons

It has recently been shown that global nucleotide excision repair is very inefficient in differentiated neurons. Human hNT neurons challenged with UV light were almost totally deficient in removing cyclobutane pyrimidine dimers (CPDs) from the bulk of their genome (Nouspikel and Hanawalt 2000), an observation that was subsequently confirmed in primary neurons (Nouspikel and Hanawalt, *unpublished*). The repair of other lesions by NER, such as those of benzo(a)pyrene diol epoxide, was also attenuated. Such a phenomenon can be possibly explained by the fact that neurons are terminally differentiated cells and their nuclear DNA is not replicated. It is conceivable that neurons might dispense with the energy expense required to remove lesions from their largely unused genome (REFS). For these cells to remain viable, it is only necessary to maintain the integrity of those genes that are expressed in neurons. The same authors have demonstrated that transcription-coupled repair (TCR) of CPDs is proficient in neurons, accounting for the integrity of transcribed genes in spite of the global deficiency in NER.

It is interesting to relate this phenomenon to recent observations in the pathology of Alzheimer's disease: that AD neurons attempt to reinitiate the cell cycle just before they degenerate (McShea et al., 1999, Raina et al., 1999). Therefore, the death of neurons in AD may be caused by an inability to complete mitosis and the life-long accumulation of DNA lesions in the non-transcribed areas of the genome.

In addition to TCR, a novel repair phenomenon termed differentiation-associated repair (DAR), was observed in neurons (Nouspikel and Hanawalt, 2000). It consists in the proficient repair of the *non-transcribed strand* in active neuronal genes. By definition, TCR only acts on the transcribed strand, while the non-transcribed strand is repaired by the same pathway that repairs the bulk of the genome. The low NER efficiency of normal neurons should thus result in the accumulation of lesions in the non-transcribed strand, a potentially dangerous situation since this strand is needed as a template to repair the transcribed strand by TCR. It is possible that DAR evolved as a special mechanism to ensure that both strands of active genes will be properly repaired in terminally differentiated cells. While the molecular mechanisms underlying DAR are unknown, several NER genes are noted to be overexpressed in neurons: XPG, XPF and ERCC1 (REF), which together form the two structure-specific nucleases responsible for incising the damaged strand to initiate NER. Whether the elevated levels of these NER enzymes suffices to explain DAR remains to be demonstrated.

Similar results have been observed in PC12 cells that were induced to differentiate into neuron-like cells (Hanawalt at al. 1992), in terminally differentiating rat myoblasts (Ho and Hanawalt 1991) and more recently in human HL60 leukemia cells differentiated towards the macrophage pathway (Nouspikel & Hanawalt, *unpublished*). This raises the possibility that this peculiar NER phenotype is not specific to neurons, but rather a common feature of terminally differentiated cells.

DNA repair in neurodegenerative disease

BER and Chronic Neurodegenerative Disease

A key feature that distinguishes the various age-related neurodegenerative disorders is the selective loss of neurons in different brain regions. For example, motor neurons in the cerebral cortex, brain stem, and spinal cord are primarily affected in ALS, whereas neuronal loss predominates in the frontal and temporal cortex and hippocampus of patients with AD. In contrast, idiopathic PD is characterized by the progressive degeneration of the nigrostriatal dopaminergic system and other subcortical

neuronal systems in association with the presence of Lewy bodies, a neuropathological hallmark of PD. Although the mechanisms responsible for the selective loss of neurons and the formation of these pathological hallmarks are unknown, the presence of DNA damage (indicated by TUNEL labeling or 8oxoG) in tangle-bearing and non-bearing neurons of AD (Nunomura et al. 1999, Anderson et al. 2000), motor neurons of the ALS cortex and spinal cord (Ferrante et al. 1997, Fitzmaurice et al. 1996), or dopaminergic neurons of the Parkinson's diseased *substantia nigra* (Zhang et al. 1999, Shimura-Miura et al. 1999) suggests that DNA repair is compromised in neurodegenerative disease. However, the pattern of oxidative damage to DNA bases differs among these neurological disorders suggesting that different aspects of oxidative DNA repair may be perturbed in neurodegenerative disease. , in AD brain tissue, thymine glycol and Fapy-adenine levels are elevated in forebrain structures (parietal, occipital and frontal lobes, hippocampus) (Gabbita et al. 1998, Lovell et al. 1999, Nunomura et al. 1999), while levels of these and other oxidative DNA adducts are lower or comparable in matching control brain tissue. In the *substantia nigra* of PD subjects, only elevated levels of 8-oxoG and reduced levels of Fapy-guanine were found in 7 out of 11 brain regions (Alam et al. 1997, Sanchez-Ramos et al. 1994). 8-OxoG is also elevated in the motor cortex and anterior horn cells of the spinal cord of ALS subjects (Ferrante et al. 1997, Fitzmaurice et al. 1996), but other oxidative DNA adducts have not been measured. In mammals, thymine glycol, Fapy-adenine and 8oxoG are repaired by different DNA glycosylases suggesting that the different patterns of oxidative DNA damage in ALS, PD and AD brain tissue may reflect disturbances in specific enzymes of the BER pathway. A reduction of OGG1 levels and activity in AD cortical and hippocampal tissue (Lovell et al. 2000) and APE in ALS (Kisby et al. 1997), PD (Kisby et al. 2000) and western Pacific ALS/PDC (Kisby et al. 1999) brain tissue are recent indications that BER is perturbed in various age-related neurodegenerative disorders. Moreover, recent studies suggest that these BER proteins may have a role in the pathological hallmarks of certain neurodegenerative disorders (e.g., AD). The co-localization of APE with paired helical filaments and amyloid fibrils in muscle tissue from elderly subjects with sporadic inclusion body myositis (Broccolini et al. 2000) is evidence suggesting the possible involvement of APE in the formation of plaques and paired helical filaments. Further work will be required to confirm these findings by determining if this BER protein (or others) plays an important role in the neurodegenerative disease process. As a cautionary note, it should be mentioned that an imbalance of one or more enzymes in BER has been shown to sometimes *protect* cells against DNA damage, to have no effect, or to increase the sensitivity. The aberrant overexpression of the cytoplasmic DNA repair protein 8-oxo-GTPase in nigrostriatal neurons of PD subjects (Shimura-Miura 1999) is an example in which brain tissue injury is correlated with increased DNA repair.

BER and Western Pacific ALS/PDC

The sporadic nature of ALS, PD and AD suggests that environmental factors also play an important role in neurodegenerative disease. A prototypical neurodegenerative disorder found in the western Pacific with features of ALS, PD and an Alzheimer-like dementia (commonly referred to as western Pacific ALS/PDC) has been the subject of an extraordinary amount of research over the past forty years. Dementia, parkinsonism, motor neuron degeneration and supranuclear palsy represent the key clinical features of the western Pacific disease, while Alzheimer-like neurofibrillary degeneration dominates the pathological picture (Hirano et al. 1958). Recent epidemiological studies indicate that exposure to cycad food (containing the genotoxin cycasin) is significantly associated with an increased risk for western Pacific ALS/PDC (Reed et al. 1987, Zhang et al. 1996), supporting the hypothesis that the cycad genotoxin plays an important role in the etiology of western Pacific ALS/PDC. Indeed, cycasin and its active metabolite methylazoxymethanol (MAM) are reported to persistently damage neuronal DNA both *in vitro* (Esclaire et al. 1999) and *in vivo* (Fischer et al. 1972) by both apoptotic (Ferrer et al. 1997a, Ferrer et al. 1997b, Lafarga et al. 1997) and nonapoptotic (Wood and Youle 1995) mechanisms. Alkylation of DNA by MAM is considered the initial event that perturbs the cell cycle (Bedford et al. 1974, Van Den Berg and Ball 1972) and triggers neuronal cell death (Matsumoto 1985, Matsumoto et al. 1972, Nagata and Matsumoto 1969). In brain and somatic tissues, MAM forms adducts with guanine (N^7-methylguanine and O^6-methylguanine)(Matsumoto and Higa 1966, Nagata and Matsumoto 1969, Nagasawa et al. 1972). In addition to its DNA damaging properties, the cycad genotoxin inhibits nervous tissue DNA repair both *in vitro* and *in vivo* (Kisby et al. 1999) and neurons appear to be the primary target. Pre-treatment of cortical explants or human SY5Y neuroblastoma cultures with pharmacological agents

that inhibit MGMT (i.e., O^6-benzylguanine) (Kisby et al. 1994, Kisby et al. 1995b) or by gene-targeting of MGMT (Kisby et al. 1997) increased their susceptibility to MAM or cycasin. Cerebral levels and activity of APE were also reduced in rats treated *in utero* with MAM (Esclaire et al. 1999). In cortical neuronal cultures, MAM-induced DNA damage was associated with reduced APE levels and an increase in tau gene expression. These studies suggest that MAM is capable of directly damaging neuronal DNA and interfering with BER of alkylated or oxidative DNA damage. It is conceivable that MAM or other environmental genotoxic agents may perturb nervous tissue DNA repair to increase the vulnerability of neurons to persistent DNA damage, altered gene expression and eventual lethality. The ability of MAM to selectively target neurons may be explained by the reported low level of DNA repair in neurons (Belloni et al. 1999, Ide et al. 2000, Kisby et al. 1999, Wilson et al. 1996, Ledoux et al. 1996). These provocative observations suggest that post-mitotic neurons that survive MAM treatment are structurally (DNA damage) and functionally (APE and tau gene expression) impaired, possibly for extended periods of time. These long-lasting changes in neurons may be relevant to understanding the molecular pathology of chronic neuronal degeneration observed in western Pacific ALS/PDC and other age-related neurodegenerative disorders.

Neurological symptoms in DNA repair disorders

There are two autosomal recessive diseases associated with deficiencies in enzymes involved in NER. Xeroderma pigmentosum (XP) is characterized by photosensitivity, poikilodermic skin lesions in sun-exposed areas together with a high incidence of skin cancers and, in some cases, neurological symptoms. There are eight complementation groups of XP, corresponding to the seven NER enzymes XPA through XPG (no patient has been found yet with an ERCC1 deficiency), and the XP-variant in which the defect is in a DNA polymerase used in translesion synthesis. At the cellular level, XP is characterized by a complete lack of NER both at the global genome level and in TCR. The only exceptions are in group C and group E that retain TCR, probably because the lesion-recognition roles of XPC and XPE are replaced by RNA polymerase II in TCR (reviewed in Hanawalt et al., 2001)).

Cockayne syndrome (CS) is another, quite different disease characterized by growth and mental retardation, neurological abnormalities, severe photosensitivity, but no increase in the incidence of skin cancer (Nance and Berry, 1992). CS cells retain NER at the global genome level, but lack TCR. It was initially thought that the lack of TCR was the cause of the disease but, if that were so, XP patients (with the exception of XP-C and XP-E) should present all the symptoms of CS, which is not the case. Current hypotheses for the molecular causes of CS include a) a subtle deficiency in transcription (Hoeijmakers, 1994, Vermeulen et al., 1994), b) a lack of recycling of the factors involved in both TCR and transcription, such as XPB and XPD which are subunits of TFIIH, (van Oosterwijk et al., 1996) or c) a lack in TCR of oxidative damage, which is proficiently repaired in XPC and XPE patients (Hanawalt, 1994, 2001; Cooper et al., 1997). There are five CS complementation groups corresponding to the two TCR-specific enzymes CSA and CSB, and to three XP enzymes: XPB, XPD, and XPG. This overlap between the two diseases may seem surprising, but it can be explained by a careful mutation analysis: some mutations always give rise to XP, others to CS (Nouspikel et al., 1997, Lehmann, 2001). Curiously, there is another rare hereditary disease, UVSS, in which there is a deficiency of TCR for UV irradiated cells, but the victims have none of the developmental or neurological problems of CS (Itoh, 1995; Spivak et al., submitted). The gene mutated in UVSS is currently unknown.

As mentioned, both XP and CS are associated with neurological abnormalities. However, the types of abnormalities are clinically and neuropathologically quite distinct. Awareness of the differences in neuropathology between these two diseases is important for potentially relating them to specific molecular deficits.

Xeroderma Pigmentosum (XP)

The early report of deSanctis and Cacchione (De Sanctis and Cacchione, 1932) of neurological abnormalities and dementia in two XP patients, which they termed "L'idiozia xerodermica" was the first indication that this disease could be associated with neurological abnormalities. Subsequent authors have used the term deSanctis-Cacchione syndrome to refer to severe neurological

abnormalities in XP patients (reviewed in Friedberg et al., 1995). Robbins (Robbins et al., 1991) introduced the term XP neurological disease (XP ND) to delineate a specific set of clinical features displayed by XP patients with neurological disease, and to distinguish these features from the neurological abnormalities observed in other hereditary DNA repair deficiency syndromes such as CS.

The early signs and symptoms of XP ND are sensorineural deafness, and hyporeflexia progressing to areflexia. Subsequent signs are vestibular and gait disturbances, motor abnormalities, abnormal EEG, cognitive difficulties, and dementia (Robbins et al., 1991). Neuroradiological studies of XP patients typically reveal cerebral atrophy and ventricular dilation (Mimaki et al., 1986). Examination of the postmortem brains of XP patients reveals gross ventricular dilation and generalized atrophy (Yano, 1950). The magnitude of the loss of brain tissue can be quite severe, with losses of approximately 40% of the brain mass.

At the cellular level, the main feature of the brain of a patient with XP ND is the loss of neurons throughout the central nervous system. Severely affected areas include the cerebral cortex, basal forebrain, and hippocampus (Yano, 1950, Roytta and Antitnen, 1986, Itoh et al., 1999) as well as the spinal cord (particularly motor neurons) and the basal ganglia. Also notable is the severe loss of monoaminergic cells in the *substantia nigra*, ventral tegmental area, and *locus coeruleus*. The loss of neurons can be so severe that the *locus coeruleus* or *substantia nigra* may be indistinct upon gross examination (Yano, 1950, Reed et al., 1969, Roytta and Antitnen, 1986,). In some ways, therefore, the neurodegeneration observed in the brains of XP ND patients encompasses the neuropathology of several major neurodegenerative disorders: the cortical atrophy observed in Alzheimer's disease, the basal ganglia degeneration of Huntington's disease, and the loss of dopaminergic neurons that are characteristic of Parkinson's disease.

While the neurodegeneration in XP ND is quite widespread, some areas of the brain are relatively spared. Of particular note are the granule cells of the cerebellum (Itoh et al., 1999). Also, as noted above, sensory nuclei of the brainstem and spinal cord are not affected as strongly as motor areas. Finally, neuropathological examination of the brains of patients with XP ND have consistently failed to identify neurofibrillary plaques or tangles, amyloid deposits, or Lewy bodies, thus supporting the classification of XP ND as a primary neurodegeneration.

XP ND is observed in XP patients in groups A, C, and D, with the majority of patients coming from group A. The precise location of the mutation within the XPA gene determines the severity of the neurological disease (States et al., 1998), and can be predictive of the age at which certain symptoms such as loss of the ability to walk will occur (Maeda et al., 1995). XP ND is also observed in some XP-D patients with mutations that give rise to XP without features of CS (Andrews et al, 1977). The presence of neurodegeneration in group C patients is often unappreciated, since it is asymptomatic. However, careful audiological studies of XP-C patients have revealed a progressive loss in the ability to hear at the high end of the audiological spectrum, consistent with the earliest signs of XP ND (Robbins et al., 1993). Patients in groups B and G develop neurological abnormalities that have the combined features of XP ND and Cockayne's syndrome. Patients in groups E, F, and V do not, as a rule, develop XP ND. While there are reports of neurological abnormalities in a small number of XP-F patients (Matsumura et al., 1998, Sijbers et al., 1998), the clinical characteristics of these abnormalities is distinct from what is observed in XP ND. Whether these particular abnormalities are the result of the specific XP-F mutations that these patients inherited, or result from some other cause and are coincidental to the XP-F mutations, remains to be determined.

Candidate neurodegenerative DNA lesions in XP

Since solar UV light cannot reach neurons of the human brain, Robbins and colleagues (Andrews et al., 1978) proposed that the neurodegeneration observed in XP patients must be due to some type of endogenous DNA damage. These authors further proposed that this hypothetical DNA damage accumulates in neurons of XP patients, resulting in interference with gene transcription and ultimately cell death. A later study by Satoh et al,(1993) demonstrated that cell lines from XP patients lack the ability to repair some class(es) of oxidative DNA lesions. Several oxidative DNA lesions were proposed by the authors as candidates for the endogenous damage that accumulates in XP neurons

and leads to cell death. One class of oxidative lesion proposed were the 8,5' cyclopurine lesions. Cyclopurines are formed via hydroxyl radical attack of DNA and the resultant 8', 5' bond produces a significant helical distortion as is typical of DNA lesions that are substrates for NER. Recent studies by Kuroaka et al (Kuraoka et al., 2000) and Brooks et al (Brooks et al., 2000) have shown that cyclopurines are substrates for NER, but are not removed by BER, a pathway that repairs most other types of oxidative DNA damage. Brooks et al (Brooks et al., 2000) also showed that a single cyclodeoxyadenosine lesion on the transcribed strand of an active gene is a strong block to gene expression in mammalian cells, a biological property consistent with causing neuronal death. Taken together, these data indicate that the cyclopurine DNA lesions are good candidates for the neurodegeneration that occurs in XP. However, further studies are necessary to directly demonstrate the role of such lesions in neurodegeneration. It will be important to demonstrate the presence of these lesions in the brains of age-matched non-neurological controls, and then elevated levels in the brains of XP patients.

In addition to the cyclopurines, there are other candidate neurodegenerative DNA lesions as well. One candidate is the malondialdehyde-deoxyguanosine lesion resulting from the attack of DNA by the lipid peroxidation product malondialdehyde (Marnett, 1999). This lesion is a known substrate for NER (Johnson and et al., 1997), and levels of this lesion are reportedly quite high in the brain (Cai et al., 1996). The influence of this lesion on gene expression in mammalian cells remains to be determined.

The other possibilities include non-bulky DNA lesions such as thymine glycol, 8oxoG, discussed earlier. These lesions are substrates for BER, but also for NER (Reardon et al., 1997). Reardon et al. (1997) have suggested that NER plays a critical backup role in the repair of these prevalent oxidative lesions, and that loss of the back-up pathway might have neuropathological consequences. Studies using knockout mice lacking the OGG1 gene, which encodes a DNA glycosylase involved in the BER of 8-oxo-deoxyguanine, are also consistent with a role for NER in removing 8oxoG (Klungland et al , 1999).

Cockayne's Syndrome (CS)

While at first glance the neurological abnormalities in XP and CS appear similar, there are important differences. Like XP patients with ND, many CS patients exhibit microcephaly (Nance and Berry, 1992), which is often more severe than that observed in XP patients. Other clinical manifestations in CS are abnormal nerve conduction and abnormal auditory evoked potentials. While some loss of reflexes is observed in severely affected CS patients, this is in contrast to the complete areflexia observed early in the progression of XP ND. Neuroradiological examination of the brain reveals ventricular dilation in CS, as also seen in XP. However, calcification of the basal ganglia is also commonly observed in CS patients (Erdem et al., 1994, Ozdirim et al., 1996), a pathological feature not found in XP ND. In addition to a calcified basal ganglia, the other major neuropathological finding in CS is a patchy dysmyelination of certain areas of the brain. Again, this pathology is not observed in the brains of XP patients. Although neuronal loss is observed in CS the anatomical pattern is quite different than that seen in XP. For example, whereas the cerebral cortex is severely affected in XP, neuronal loss is not observed in the cortex of CS patients (Itoh et al., 1999). Likewise, in contrast to the dramatic loss of dopamine and norepinephrine containing neurons in XP, these neurons are reportedly spared in CS (Soffer et al., 1979, Leech et al., 1985, Itoh et al., 1999). On the other hand, neuronal loss in the cerebellum, particularly in the granule cell layer, is much more severe in CS than in XP (Itoh et al., 1999).

Candidate DNA lesions in CS

As noted above, CS patients lack the capacity to remove several types of oxidative DNA damage from the transcribed strand of active genes (Cooper et al., 1997, Leadon and Cooper, 1993). A recent study by LePage et al. (2000) demonstrated that a single unrepaired 8-oxoG on the transcribed strand of an active gene was a complete block to transcription in CS cells. Therefore the inability to remove transcription blocking oxidative lesions such as 8-oxoG from active genes may be an important molecular mechanism to explain some of the clinical manifestations of CS, such as dwarfism, progeria and some neurological abnormalities. However, it is less clear how this defect would result in the

specific neurological abnormalities observed in CS described above, i.e. the patchy demyelination and calcification of the basal ganglia that occurs in CS but not in XP patients.

In addition to the pathology described above, there are some reports of highly abnormal cells in the CS brain, including astrocytes with large, abnormally shaped nuclei, and some binucleated neurons (Soffer et al., 1979, Leech et al., 1985). Leech et al (1985) pointed out that the morphology of these cells was similar to that of cells that had been exposed to high doses of ionizing radiation. As ionizing radiation produces damage to cells via the generation of hydroxyl radicals, unrepaired oxidative DNA damage may be responsible for the abnormal morphology of these cells.

Animal models for BER deficiency

There is no known human disease due to a deficiency in the BER pathway, most probably because such a phenotype would be lethal at an early stage of development. Therefore, animal models become an important tool for studying the neurological consequences of BER impairment. Since most of the BER genes have been 'knocked out' (or 'in') in mice, these mutant mice have become important models for exploring the significance of different types of DNA damage in neurodegenerative disease. For the most part, nervous tissue of these mutant mice have not been thoroughly examined for DNA damage and DNA repair. The evident lack of interest may stem from the failure to document that these mutant mice develop overt neurodegeneration comparable to that seen in human neurological disorders. However, recent studies indicate that the nervous system of DNA repair mutant mice may be particularly vulnerable to DNA damage that is commonly found in neurodegenerative disease. As our knowledge of the molecular neuropathology of neurodegenerative disorders increases, mouse models will likely become important tools for characterizing the functional importance of DNA repair in the nervous system and especially its role in the diseased brain.

Mice that are defective in APE or DNA Pol ß, two pivotal proteins of the BER pathway (Evans et al. 2000, Srivastava et al. 1998), are useful animal models to explore the role of oxidative DNA damage in neurodegenerative disease. In either case, these mice fail to thrive during development (i.e., embryonic lethal at 10.5 post-coitus) suggesting that both of these BER proteins play an important role in maintaining the integrity of cells. More importantly, cultures of embryonic fibroblasts or cerebellar neurons or tissues from mice with heterozygous mutations for these BER proteins have recently been shown to be very sensitive to both alkylating agents and agents that induce oxidative stress (e.g., menadione) (Ochs et al. 1999, Sobol et al. 2000, Kisby et al. 1998, Meira et al. 2001). Such cultures from an APE heterozygote deficient mice were reported to be sensitive to the redox-cycling agent menadione and the pesticide paraquat (Kisby et al. 1998, Meira et al. 2001). In addition, these mice exhibited elevated levels of total serum lipid hydroperoxides and F2-isoprostanes, markers of oxidative stress (Meira et al. 2001). These results are consistent with a proposed role for APE in protecting nervous and non-nervous tissue from oxidative stress induced injury. It is conceivable that humans with heterozygous mutations in the homologous *HAP1* gene may be at an increased risk for oxidative stress injury of nervous tissue. The recent identification of such variants of the *HAP1* gene in the human population is consistent with this hypothesis (Hadi et al. 2000, Hu et al., 2001).

Summary and conclusions

Neurons, and probably other terminally differentiated cells, have low levels of several enzymes involved in DNA repair and are, therefore, inefficient at repairing their DNA rendering these cells more sensitive to certain types of DNA damage. Indeed, an accumulation of DNA damage has been observed with aging and in several neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease and Parkinson's disease. Although the primary cause of these neurological disorders probably resides elsewhere, the accumulation of DNA damage is likely to contribute to, if not initiate, the neurodegenerative process. This conclusion is supported by the presence of features that are typical of neurodegenerative disease in hereditary disorders due to a lack of DNA repair (i.e. xeroderma pigmentosum and Cockayne's syndrome) and in animal models in which ~~a~~ DNA repair ~~pathway~~ has been artificially impaired. It is clear from this brief review of DNA repair in the nervous system that much remains to be determined, especially with respect to the molecular mechanism(s) responsible for the neurodegeneration in inherited DNA repair disorders and whether similar processes are abnormal in neurodegenerative disease. Future studies that use various animal models

with defects in one or more DNA repair pathways may reveal the importance of DNA repair in neurodegenerative disease.

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